Establishment and characterisation of new human induced pluripotent stem cell lines and cardiomyocyte differentiation – a comparative view

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Abstract

Research background and aims. The aim of this study was to establish and characterise iPSC-lines generated with two different methods, as well as to differentiate the created cells into cardiomyocytes, maintaining a comparative view. Since traditional culture conditions include xenogenic and undefined components, also an experiment on establishing and maintaining iPSCs feeder-free was conducted. In addition to studying the reprogramming efficiency, also the expression of pluripotency genes was studied quantitatively at mRNA level.

Materials and methods. iPSCs generated from patient fibroblasts were characterised by studying the expression of exogenous and endogenous pluripotency genes by PCR an RT-PCR, staining the cells with pluripotency markers, karyotyping and an embryoid body *in vitro* -differentiation potential assay, and RT-PCR to detect markers for each germ layer. The cardiomyocyte differentiation was performed in co-culture with END-2 cells. Pluripotency gene expression was also studied with real-time qPCR at passages 3 and 9.

Results. All studied iPSC-lines except one Geltrex®-line lost at p. 9 showed successful reprogramming with no qualitative differences between sendai-virally or episomally reprogrammed lines. The lines that were cultured feeder-free stained positive for neural markers, and differentiated, neural precursor-like cells were present at all passages, which was not encountered for MEF-cultured lines. For the two cardiac-differentiated lines, the efficiency of differentiation assessed in two ways showed a more efficient differentiation of the sendai-virally reprogrammed line than the one reprogrammed with episomal plasmids. Gene expression studies showed no significant changes in pluripotency gene expression between lines or passages except for the gene NANOG, the expression of which was lower in the later passage than the earlier passage. The reprogramming efficiencies observed were extremely low, in the range of 0,005–0,017%.

Conclusions. Although stem cell research is trying to generate feeder-free and xeno-free methods for iPSC generation and maintenance, the method tested in this thesis did not possess real advantages when compared to the MEF-culturing. The reprogramming efficiencies between feeder-free or MEF-cultured lines derived episomally did not differ. The pluripotency genes were already highly expressed in early passage iPSCs. The differences in pluripotency gene expression between early and late passages were small. Cardiac differentiation was more efficient for sendai-virally reprogrammed line compared to episomally differentiated line. However, more lines would be needed to verify these results.

Key words induced pluripotent stem cell (iPSC), mouse embryonic feeder (MEF), cardiomyocyte, cardiac differentiation, reprogramming efficiency, differentiation efficiency, episomal plasmid

PRO GRADU –TUTKIELMA

Paikka:	TAMPEREEN YLIOPISTO Lääketieteen ja biotieteiden tiedekunta
Tekijä:	MARTTILA, SUVI TUULI
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Tiivistelmä

Tutkielman tausta ja tavoitteet. Tämän Pro Gradu -työn tarkoituksen oli luoda ja karakterisoida kahdella eri menetelmällä uusia indusoituja pluripotentteja kantasolulinjoja, sekä erilaistaa niitä sydänlihassoluiksi END-2-erilaistusmenetelmällä vertailevalla otteella. Koska perinteiset soluviljelymenetelmät sisältävät eläinperäisiä soluja sekä tuntemattomia tekijöitä, tutkittiin myös soluvapaan Geltrex®-matriisin ja mTeSR1- kasvatusmediumin soveltuvuutta indusoitujen kantasolujen luontiin ja ylläpitoon. Lisäksi tutkittiin uudelleenohjelmoinnin tehokkuutta sekä pluripotenssigeenien aktivoitumista uudelleenohjelmoinnin alkuvaiheessa.

Tutkimusmenetelmät. Luotuja kantasolulinjoja kasvatettiin yhteisviljelmissä MEF-solujen kanssa ja linjat karakterisoitiin tutkimalla eksogeenisten ja endogeenisten pluripotenssigeenien ilmentymistä PCR:n, RT-PCR:n ja määrällisesti real-time qPCR:n avulla, sekä proteiinitasolla immunovärjäämällä pluripotenssiproteiineja. *In vitro* -erilaistumista tutkittiin embryoid body-menetelmällä sekä tunnistamalla niistä RT-PCR:n avulla eri alkion kerrosten läsnäolo. Sydänerilaistus suoritettiin yhteisviljelmässä END-2 solujen kanssa.

Tutkimustulokset. Kaikki tutkitut linjat yhtä Geltrex®:llä kasvatettua linjaa lukuun ottamatta todettiin uudelleenohjelmoituneiksi karakterisointien perusteella. Sendai-virusmenetelmällä luotu solulinja erilaistui tehokkaammin sydänlihassoluiksi kuin episomaalisilla plasmideilla uudelleenohjelmoitu solulinja. Soluvapaalla alustalla kasvatetut kantasolulinjat erilaistuivat spontaanisti MEF-yhteisviljelmissä kasvavia iPS-soluja enemmän, ja ilmensivät alkeellisille hermosoluille tyypillisiä proteiineja. Uudelleenohjelmoinnin tehokkuus kaikille linjoille oli matala, 0,005–0,017 %. Pluripotenssigeeniekspressiossa ei potilaiden tai eri aikapisteiden välillä havaittu merkittäviä muutoksia kuin yhdelle geenille, NANOG:lle, jonka ilmentyminen myöhemmässä vaiheessa oli alhaisempi kuin aikaisemmassa aikapisteessä.

Johtopäätökset. Verrattaessa perinteistä viljelymenetelmää yhteisviljelmissä eläinperäisten MEF-solujen kanssa, tässä lopputyössä testatussa soluvapaassa menetelmässä ei saavutettu suuria etuja vaan niissä havaittiin suuria määriä erilaistuneita hermosolujen esiasteita. MEF-yhteisviljelmissä sekä soluvapaalla Geltrex®-matriisilla uudelleenohjelmoitujen iPS-solujen erilaistumistehokkuudet eivät eronneet merkittävästi toisistaan. Pluripotenssigeenit aktivoituvat jo aikaisessa vaiheessa ja ilmentymistasojen vaihtelut olivat alhaisia. Sendai-virusmenetelmällä luotu iPS-solulinja erilaistui tehokkaammin sydänlihassoluiksi kuin plasmideilla luotu iPS-linja. Koska tulokset koostuivat vain kahden linjan vertailusta, useampia linjoja tarvitaan tulosten varmistamiseksi.

Avainsanat indusoitu pluripotentti kantasolu (iPS-solu), hiiren alkion fibroblasti (MEF), episomaalinen plasmidi, sydänlihassolu, sydänerilaistus, uudelleenohjelmointitehokkuus, erilaistustehokkuus

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[1]Manzini S, Viiri LE, Marttila S, et al. A Comparative View on Easy to Deploy non-Integrating Methods for Patient-Specific iPSC Production. Stem Cell Rev 2015;11:900-8.

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ABBREVIATIONS

AA	Ascorbic Acid
AFP	Alpha-fetoprotein
AZA	5-aza-deoxycytidine
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
EB	Embryoid Body
ESC	Embryonic Stem Cell
END-2	Mouse Visceral Endoderm-like
EHS	Engelbreth-Holm-Swarm
EBNA	Epstein-Barr Nuclear Antigen
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
hTERT	Human Telomerase Reverse Transcriptase
iPSC	Induced Pluripotent Stem Cell
KLF	Kruppel-like Factor
KSR	Knock-out Serum Replacement
MAP	Mitogen-associated Protein
MEF	Mouse Embryonic Fibroblast
MEA	Microelectrode Array
MEK	Mitogen-activated Kinase Kinase
MYC	Myelocytomatosis Viral Oncogene Homolog
NDS	Normal Donkey Serum
NEAA	Non-essential Amino Acid
OCT	Octamer-binding Factor
PBS	Phosphate-buffered Saline
PAX	Paired-box Gene
REX	Reduced Expression
SOX	Sex Determining Region Y-box 2
SSEA	Stage-specific Embryonic Antigen
TRA	Tumor-related Antigen
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
WNT	A Mammalian Ortholog of the Wingless Gene Observed In *Drosophila*

1. Introduction

The discovery of Yamanaka and Takahashi in 2006 that somatic adult cells could be reprogrammed back into a pluripotent state by introducing four distinct transcription factors (Takahashi and Yamanaka, 2006) changed the frame in which stem cell research is now conducted. They named these stem cells induced pluripotent stem cells (iPSCs). iPSCs are embryonic stem cell (ESC)-like cells that are able to differentiate into cells of all the three germ layers, i.e. into all cell types except the extra-embryonic tissues. Previously research had focused on studying the embryonic stem cells. Because of their limited availability (in Finland for example available only from non-implantable embryos derived for fertility treatments) and ethical considerations, the generation of iPSCs revolutionised the research. Now pluripotent stem cells from any individual and multiple cell types could be obtained. Since the emergence, the first steps included the generation of first human iPSCs (Takahashi et al., 2007; Yu et al., 2007) and verification of the ESC-like pluripotent state. The iPSCs have indeed been established to be equivalent to ESCs morphologically, functionally, epigenetically and transcriptionally (Maherali et al., 2007; Mikkelsen et al., 2008; Okita, et al. 2007; Takahashi et al., 2007; Wernig et al., 2007).

IPSCs have many uses. As such they can be used to study developmental biology, a subject that only little is known of since human embryonic development is challenging to study. As they can theoretically be differentiated into any cell type, *in vitro* -disease models for modelling of diseases can be made. These models can also be used for drug and toxicity screening, offering more insight to drug safety than is obtained with animal studies only. The iPS-research is now focused on finding the best generation methods, cell types, factors and culture conditions to obtain high-quality iPSCs (Brouwer et al., 2016).

Cardiac differentiation methods have been generated already for human ES-cells and later adapted to differentiate iPS-cells. Ultimately, the differentiated cardiomyocytes could possibly be used in the repair and regeneration of cardiac tissue (Batalov and Feinberg, 2015). However, this goal is still far away. Currently, the differentiated cardiomyocytes can be used for disease modeling, drug testing and toxicity screening. Moreover, as patient-specific lines can be generated, lines from patients with various genetic cardiac disorders can and have been created (Terrenoire et al., 2013). The main research areas in the field are the development of more

effective *in vitro* -differentiation protocols, guidance of differentiation into special subtypes and methods to isolate them. Since cardiomyocyte obtained by differentiation of iPSCs express an immature, more fetal-like phenotype, the research is also focusing on generating cardiomyocytes of higher maturity. (Rajala et al., 2011)

2. Literature review

2.1 Stem cells

Stem cells are functionally undifferentiated cells possessing two key properties: they have the capacity to self-renew, and to differentiate into specialised cell types (Weissman et al., 2001; Smith, 2001). Self-renewal means that the cells can divide extensively, maybe even indefinitely, giving rise to identical undifferentiated daughter cells. In addition, these cells can also differentiate into at least one or multiple different cell types. During differentiation, the stem cell divides producing two daughter cells, of which the other differentiates and the other remains a stem cell. Two types of mechanisms for this are proposed: the first possibility is that the stem cell divides asymmetrically giving rise to two cells with a different complement of proteins. The other possibility is that the differentiation of the other daughter cell is caused by external signals: the daughter cell that does not differentiate occupies a specific stem cell niche and stays undifferentiated, while the other ends up outside the stem cell niche and differentiates. In many cases, both mechanisms may apply. (Wolpert et al., 2011)

Stem cells can be classified according to their differentiation ability. During embryonic development in mammals, the fertilized egg possessed the ability to differentiate into all cell types in an individual, as well as extra-embryonic tissues, and is called totipotent. As the fertilized egg divides further, it forms the compacted morula, in which individual cell outlines are no longer visible. The insides of the morula form the inner cell mass seen at the later-stage blastocyst. The outer layer of the blastocyst gives rise to the trophectoderm, from which extraembryonic tissues placenta, umbilical cord and fetal membranes are later formed, while the inner cell mass gives rise to the embryo proper. All three germ layers - endoderm, mesoderm and ectoderm – are formed from the inner cell mass and they have the ability to differentiate into all cell types and tissues encountered in an individual. However, no extra-embryonic tissues can form from the inner cell mass, and the cells are referred to as pluripotent. (Wolpert et al., 2011)

As embryonic development gradually proceeds, the cells tend to lose their differentiation potential as they become more committed. Nonetheless, stem cells can still be found in various, but usually small, amounts in all adult tissues, where they are responsible for tissue renewal and repair (Wolpert et al., 2011). Depending on the tissue and stem cell, these adult stem cells can be either multipotent – capable of differentiating into more than one different cell types –

or unipotent, that can differentiate into a single cell type. For example, hematopoietic stem cells of the bone marrow are multipotent and can differentiate into all blood cells, whereas keratinocytes mature from unipotent stem cells in the deepest layer of the epidermis (Wolpert et al., 2011). Some debate has been going on as to whether the unipotent stem cells can be classified as stem cells, thus they are also often referred to as precursor cells (Melton, 2014). The different stem cell types are depicted in **Figure 1**.

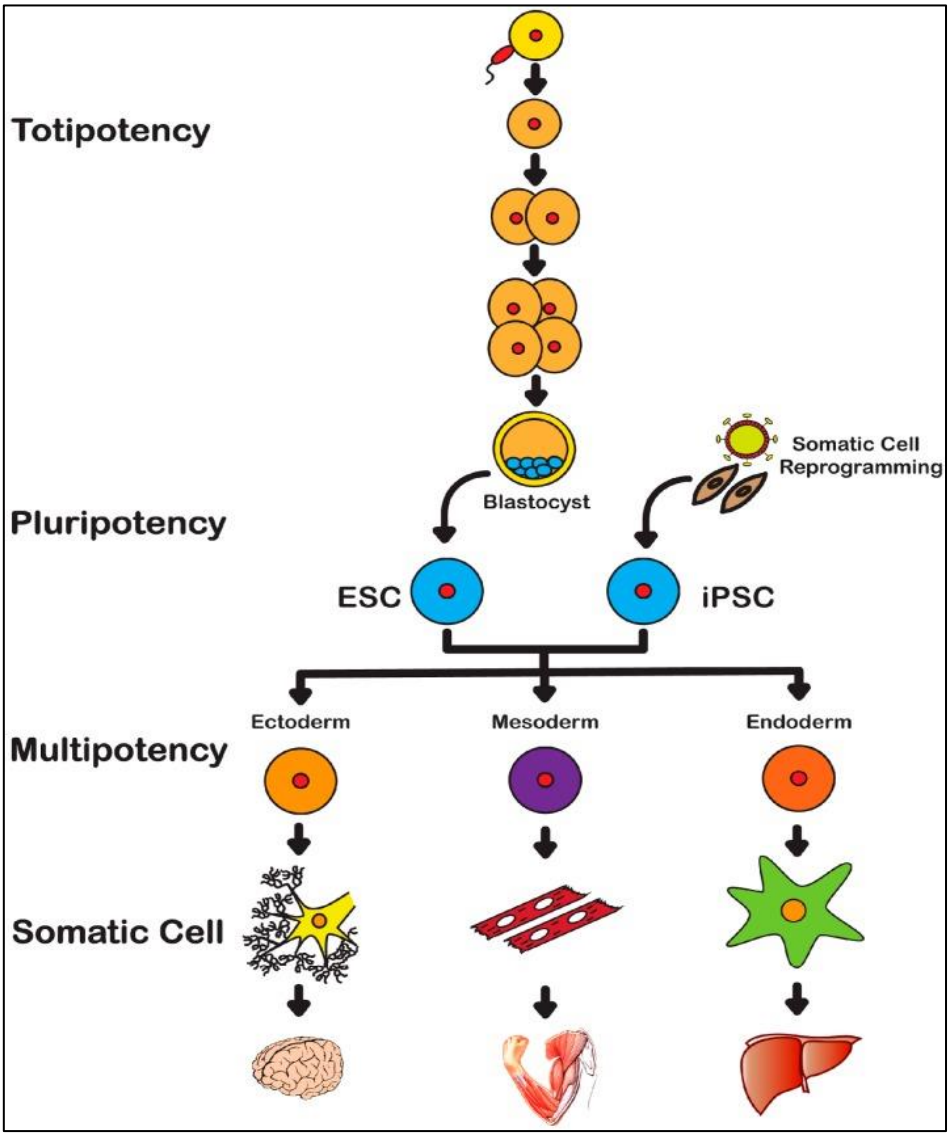

Figure 1. Classification of stem cells according to their differentiation ability. *Totipotent* stem cells of the fertilized egg can differentiate into all different cell types. Later on in the embryonic development, the inner cell mass of the blastocyst contains *pluripotent* stem cells able to differentiate into all other cell types except placental, umbilical cord- or extraembryonic membrane tissues. *Pluripotent* stem cells differentiate into cells of all three germ layers, which contain *multipotent* stem cell able to differentiate into multiple different cell types of a certain lineage, and ultimately form terminally differentiated cells that form the majority of adult tissues. (Menon et al., 2016)

The first embryonic stem (ES) cell lines were isolated from the inner cell mass of a mouse blastocyst, and were successfully maintained in *in vitro* -cultures (Evans and Kaufman, 1981; Martin, 1981). In the following years, many groups reported maintenance of undifferentiated, pluripotent embryonic stem cells from various origins in *in vitro* -cultures, and generation of differentiation protocols (Amit and Itskovitz-Eldor, 2002), that paved the way for the future discovery that revolutionised the stem cell research. Before it was thought that once the stem cell is committed into a specific lineage, it cannot differentiate into cells of another lineage. This thought has been compromised since and it has been shown that fully differentiated cells can transdifferentiate into another cell type, or not yet differentiated, but committed progenitor cells transdeterminate into another lineage (Wolpert et al., 2011). The most radical finding happened in 2006 when Takahashi and Yamanaka proved that fully differentiated cells could be reprogrammed back to the pluripotent state, introducing the concept of induced pluripotent stem (iPS) cells for the first time (Takahashi and Yamanaka, 2006).

2.2 Induced pluripotent stem cells

iPS-cells are pluripotent stem cells that can be produced from terminally differentiated cells, also from adult somatic cell by reprogramming. By introducing certain reprogramming factors into the cells, the cells dedifferentiate into a pluripotent, embryonic stem cell (ESC)-like state. (Takahashi and Yamanaka, 2006) Yamanaka and colleagues were able to produce mouse iPS-cells from mouse skin fibroblast by retroviral transduction of four transcription factor genes found to be upregulated in ESCs coding for octamer-binding factor (Oct4), sex determining region Y-box 2 (Sox2), myelocytomatosis viral oncogene homolog (c-Myc) and Kruppel-like factor 4 (Klf4), called the Yamanaka-factors or OSKM. From the resulting cells, they were able to isolate and expand the reprogrammed iPS-cells. A year later the generation of human iPS-cells was reported for the first time (Takahashi et al., 2007; Yu et al., 2007).

Because of their potential to be differentiated theoretically into almost any cell type, iPS-cells have many possible applications including drug and toxicity screening, disease modeling, cell transplantation therapies and regenerative medicine. Moreover, the use of iPS-cells circumvents ethical issues related with ES-cell (Gonzalez et al., 2011; Seki and Fukuda, 2015). The use of autologous iPS-cells for cell therapies was thought to overcome problems regarding immune reactions caused by allogenic ES-cells, but has been compromised since 2011 when Zhao et al. reported immune responses in mice receiving syngeneic iPS-transplants (Zhao et al., 2011). This is thought to be contributed by genetic and epigenetic changes that occur randomly during

reprogramming (Doi et al., 2009; Kim et al., 2010; Polo et al., 2010), as well as by the immaturity of *in vitro* –differentiated cells and possibly xenogeneic or non-physiological components used in iPS-culture (Martin et al., 2005; Tang and Drukker, 2011). More recent studies have also reported negligible or no immune responses when using iPSCs produced with an integration-free method (Guha et al., 2013; Lu et al., 2014).

Ever since the emergence of iPS-cells they have been studied extensively. The major problems concern low reprogramming efficiency, tumorigenic potential and genomic instability. The transcription factors Oct4 and Klf4 are known oncogenes, raising safety issues especially with clinical use. Viral reprogramming methods can cause genomic integration resulting in insertional mutagenesis, or result in incomplete silencing of the transgenes. The research has mainly been focusing on improving the iPS-technology by finding the most suitable cell sources, factors and methods for reprogramming, as well as development of optimal culture conditions to maintain the pluripotency of the generated iPS-cells. (Brouwer et al., 2016)

2.2.1 Cell types for reprogramming

Before reprogramming, a suitable cell type must be chosen. The cells should be easily obtained and susceptible to reprogramming, and preferably storable by freezing (Brouwer et al., 2016). Since reprogramming efficiencies are generally low, the cell source must be easily expandable to obtain enough cells for reprogramming. However, obtaining fibroblasts which are the cell type most often used for reprogramming, is an invasive procedure. More easily obtainable cells, such as cells from urine samples or cord blood cells have also been reprogrammed. Another advantage regarding the use of cord blood cells is that as immature cells they probably contain less somatic mutations and can be epigenetically easier to reprogram than adult cells, and could be stored in blood banks for later use. (Brouwer et al., 2016)

The first reprogramming was performed with fibroblasts using the Yamanaka-factors (Takahashi and Yamanaka 2006), but the type of the somatic cell used also affects the transcription factors needed for successful reprogramming. For example, neural progenitor cells or melanocytes having high endogenous expression of SOX2 can be reprogrammed without SOX2 or even with OCT4 alone (Eminli et al. 2008; Kim et al 2008; Utikal et al., 2009a). The use of less reprogramming factors, however, usually also has an effect on reprogramming efficiency (Lai et al., 2011).

It has been shown that even after reprogramming, the iPSCs elicit an epigenetic memory of the original donor cell characterised by gene expression patterns and DNA methylation (Kim et al., 2011; Bar-Nur et al., 2011; Marchetto et al., 2009; Ohi et al., 2011). Because of this, upon differentiation the iPSCs tend to differentiate more easily into cells of the same germ layer as the original donor cell (Kim et al., 2011; Bar-Nur et al., 2011). Thus, choosing a cell type from the same germ layer as the generated iPSCs will be differentiated to, can help to improve differentiation efficiency (Brouwer et al., 2016). As also demonstrated by Ohi et al., the silencing of donor cell type genes can be insufficient for many genes upon reprogramming (Ohi et al., 2011). As for improving differentiation efficiency, also the quality of the iPSCs will be improved by choosing a donor cell type of close origin to the one it will be differentiated to (Brouwer et al., 2016).

The epigenetic profile in the created iPSCs is an important characteristic separating iPSCs and ESCs. In an example study by Hiler et al. iPSCs generated from rod photoreceptor cells differentiated more efficiently into the retinae than did ESCs (Hiler et al., 2015). On the other hand, differentiating cells into cells of another germ layer than the original cell types probably results in efficiencies lower than with the use of ESCs. However, the epigenetic, gene expression and differentiation potential differences between iPSCs and ESCs seem to be diminished during passaging of the iPSCs (Chin et al., 2009; Polo et al., 2010; Nishino et al., 2011). As a result, the iPSCs are thought to lose the characteristics of the paternal cell type over time (Brouwer et al., 2016). So far most cell types used for reprogramming have been from a mesodermal origin, such as fibroblasts, adipose stem cells, dental pulp cells, cells from the hematopoietic lineage and urinary cells. Although more rarely, also cells form endodermal and ectodermal origins such as keratinocytes, hepatocytes, melanocytes and neural progenitor cells have been reprogrammed successfully. (Brouwer et al., 2016)

2.2.2 Reprogramming factors

Currently, there are many existing factors (or combination of factors) that can be used for reprogramming. Many of the factors inducing reprogramming are factors that are normally expressed in early embryos and are important for the maintenance of pluripotency in the embryo (Gonzalez et al., 2011). The original reprogramming cocktail used by Takahashi and Yamanaka in 2006 consisted of four transcription factors OCT3/4, SOX2, KLF4, and C-MYC, all factors found to be upregulated in ESCs (Takahashi and Yamanaka 2006; Takahashi et al., 2007). The efficiency of reprogramming occurred at an efficiency of 0,02% with adult human dermal

fibroblasts. These Yamanaka-factors are also the most common factors used for reprogramming (Seki and Fukuda, 2015). A bit later, another research group was reprogramming cells using a combination of transgenes SOX2, OCT4, NANOG and LIN28 (Yu et al., 2007).

Since C-MYC is a known oncogene in humans, alternative methods omitting it from the Yamanaka-factor-cocktail also managed to achieve successful reprogramming, although with a much lower efficiency (Nakagawa et al., 2008; Wernig et al., 2008). On the other hand, adding proliferation-inducing human Telomerase reverse transcriptase (hTERT) and SV 40 large T antigen to the reprogramming cocktail an efficiency of up to 0,25% could be achieved with adult fibroblasts (Park et al., 2008). Adding UTF1 or SALL4, both transcription factors associated with pluripotency, with the Yamanaka-factors also resulted in more colonies than with the Yamanaka-factors alone (Gonzalez et al., 2011). As already described earlier, the cell type used for reprogramming also affects which factors need to be used.

In addition to the actual reprogramming factors, also various facilitating compounds enhancing the efficiency of reprogramming can be added. For example, inhibition of the cell-cycle regulator mitogen-activated kinase kinase (MEK) results in enhanced reprogramming. Inhibition of reprogramming barriers such as cell senescence or apoptosis can also enhance reprogramming. Inhibition by short hairpin RNAs or knockout alleles of p53 or members of the same pathway resulted in increases in both speed and efficiency of reprogramming when compared to the use of Yamanaka-transcription factors alone. (Gonzalez et al., 2011) Other non-coding RNAs usually targeting the transforming growth factor beta (TGFβ) -pathway can be used to enhance reprogramming. A combination of microRNAs has also been used to achieve successful reprogramming without the use of transcription factors at a higher efficiency. (Anokye-Danso et al., 2011)

The use of small molecules to enhance the rate-limiting step of chromatin remodeling has been shown to increase efficiency. The most used small molecules in reprogramming protocols are histone deacetylase inhibitors valproic acid and sodium butyrate. (Malik and Rao, 2013) Recently, reprogramming of mouse cells using only small molecules was achieved (Hou et al., 2013). However, the results have not yet been demonstrated using human cells. The advantage using small molecules is that they don’t require any specific delivery method to enter the cell and can be administered at very specific amounts, making the process easier. However, the non-specific effects can cause cellular toxicity. (Brouwer et al., 2016)

2.2.3 Culture conditions

The culture conditions used for iPSC-reprogramming and maintenance are based on hESC-culture conditions developed over the past decade (Chen et al., 2011). The iPSCs can be cultured either in colonies, non-colony monolayers or as suspension cultures. The most commonly used method is a colony-based feeder cell culture usually by co-culture with mouse embryonic fibroblast (MEF) feeder cells. The supportive cells secrete growth factors necessary for survival and maintenance as well as proliferation of human pluripotent stem cells. Traditional media are based on fetal calf or bovine serum replacement supplemented with β-fibroblast growth factor (FGF). However, these traditional methods contain xenogenic cells and/or compounds, batch-to-batch variation in the biological media compounds and the fact that the factors secreted by the feeder cells remain unknown, poses safety issues. Especially for clinical use the iPSCs need to be cultured in fully defined, xeno-free conditions. (Brouwer et al., 2016) As a result, research has been focusing on the development on novel cell-free or totally xeno-free matrices and xeno-free media (Seki and Fukuda, 2015).

While human feeder cells avoid the problem of xenobiotics, the high cost and difficulties in upscaling the production has let researchers to explore other options. Matrigel is probably one of the most used cell-free matrices used to generate and maintain iPSCs in non-colony type monolayer cultures. It also has the ability to increase cell viability and proliferation when compared to traditional colony-based culture with feeder cells. (Chen et al., 2012) Geltrex® is another cell-free matrix used for culture of iPSCs (Wagner and Welch, 2010) and both are derived from the murine Engelbreth-Holm-Swarm tumor. Also totally xeno-free matrices such as Cellstart, vitronectin, laminin, recombinant proteins and various synthetic matrices have been tested (Bergrström et al., 2011; Ausubel et al., 2011; Chen et al., 2011; Miyazaki et al., 2012; Rodin et al., 2010; Mei et al., 2010; Lu et al., 2012). By suspension culture, the need for a matrix surface can be completely avoided (Zweigerdt et al., 2011), however shear forces can cause damage to the cells (Serra et al., 2012).

To remove the serum-based products with batch-to-batch variation from the media, knockout serum replacement (KSR) medium has been developed and is now largely used in iPSCs generation in feeder cultures (Seki and Fukuda, 2015). Also, a chemically defined serum-free medium called mTESR1 was developed and is now the most widely published feeder-free medium for ESC- and iPSC cultures (https://www.stemcell.com/mtesr1.html, cited 7.5.2017). Although these are improvements compared to the traditional culture media, both contain

xenogenic compounds. Multiple completely xeno-free defined media such as Essential 8 medium (E8), TeSR2 medium and Nutristem XF/FF medium are also available for successful generation of iPSCs (Chen et al., 2011; Bergström et al., 2011; Sugii et al., 2010). The benefits of especially E8 medium is also the lower cost, and the fact that the medium is composed of only 8 defined components (Chen et al., 2011).

As well as the actual matrix and media used, also other factors can enhance reprogramming efficiency or cell survival in culture. Reprogramming in hypoxic conditions of 5% O_2 rather than atmospheric 21%, increases the reprogramming efficiency 5-fold in both mice and human cells. When valproic acid is additionally used, the efficiency with mice cells increased as much as 200-fold. (Yoshida et al., 2009) L-Ascorbic acid (AA) has also been proven to promote iPSC growth and survival (Chen et al., 2011), in addition to various other small molecules presented in **Table 1**. Small molecules or hypoxia can be used to enhance reprogramming efficiency and help to improve the reprogramming of recalcitrant somatic cells. Yet another alternative would be to use embryonic stem cell -conditioned medium to induce reprogramming. (Malik and Rao, 2013)

Table 1. Small molecules and their targets to improve iPSC reprogramming efficiency (Modified from Malik and Rao, 2013)

Treatment	Process affected
Valproic acid	Histone deacetylase inhibition
Sodium butyrate	Histone deacetylase inhibition
PD0325901	MEK inhibition
A-83-01	TGFβ-inhibition
SB43152	TGFβ-inhibition
Vitamin C	Enhances epigenetic modifiers, promotes survival by antioxidant effects
Thiazovin	ROCK inhibitor, promotes cell survival
PS48	P13K/Akt activation, promotes glycolysis
5% Oxygen	Promotes glycolysis

2.2.4 Reprogramming methods

After deciding on the cell type, reprogramming factors and culture conditions to be used, a suitable reprogramming method should be picked. The method of choice should also be considered by the downstream application, for example if aiming for clinical application of the produced cells, a foot-print free method of generating iPS-cells needs to be used. In regards, the reprogramming methods can be divided into two major classes: integrating and non-integrating depending on whether the reprogramming factors are incorporated into the host cell genome during reprogramming or not (Gonzalez et al. 2011). Higher-quality iPSCs are produced by non-integrating methods since no danger of the reactivation of the pluripotency genes or insertional mutagenesis is present. Various reprogramming methods have been developed, and are outlined in **Figure 2**.

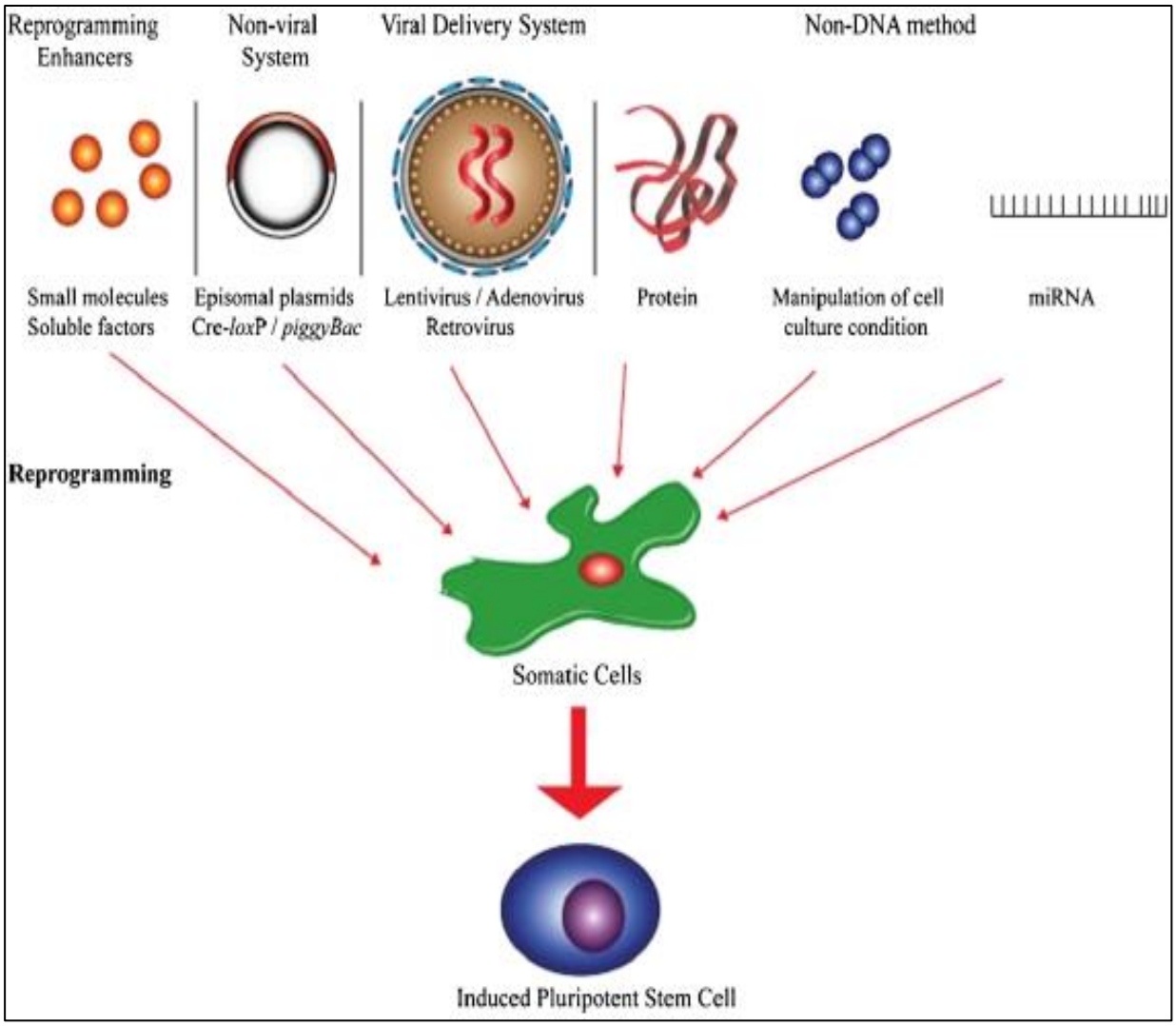

Figure 2. Methods for generating induced pluripotent stem cells. (Lai et al., 2011)

2.2.4.1 Integrating viruses

The first successful reprogramming reported used a retroviral transduction method (Takahashi and Yamanaka 2006) using Moloney murine leukaemia virus (MMLV)-derived retroviruses such as pMXs, pLib12 or pMSCV. These can infect dividing cells at an efficiency of even 90% (Gonzalez et al. 2011). Nonetheless reprogramming efficiencies using the Yamanaka-factors reported for human cells is between 0,01-0,02% (Menon et al., 2016). Another retroviral method used is transfection with lentiviruses derived from the human immunodeficiency virus (HIV). They have a higher infection efficiency and cloning capacity than the MMLV-retroviruses. As it can infect both non-dividing and dividing cells, it soon became a more preferred method for generating iPSCs over the MMLV-retroviral method (Malik and Rao, 2013). The higher efficiency has been reported to be between 0,1-2% (Gonzalez et al. 2011).

Originally several different retroviruses all containing one reprogramming factor were generated. To achieve complete reprogramming the transfected cell needs to obtain each transcription factor from different retroviruses. This may lead to uneven stoichiometric quantities of the transcription factors in the cells, and low reprogramming efficiency since the cell may not obtain all transcription factors. Moreover, the major downside using retroviruses is that the viral transgenes have been reported to integrate randomly into the iPS-cell genomes, which may cause dysregulation of proto-oncogenes and insertional mutagenesis in the host cell genome (Gonzalez et al., 2011). With the use of multiple transcription factors also the risk of insertional mutagenesis increases. The other disadvantage with retroviruses concerns gene silencing. To achieve full reprogramming, the viral transgenes need to be silenced after iPSC-formation (Hotta and Ellis, 2008), which is sometimes inefficient. Some genes may not even be silenced at all, and as the transgenes remain in the host cell genome they may be reactivated later point (Brouwer et al., 2016; Hu, 2014; Toivonen et al., 2013).

The safety issues regarding retroviruses have been addressed by creating polycistronic lentiviruses. These contain all transcription factors in one vector, separated by self-cleaving 2A peptide sequences (Brouwer et al., 2014; Carey et al., 2009). This decreases the risk of insertional mutagenesis since fewer integration sites are introduced into the genome. Moreover, drug-inducible promoters have been created to establish a controlled expression of viral transgenes, as well as controlled silencing (Hockemeyer, 2008). Also, excisable lentiviruses utilizing the CreLoxP-system have been created (Sommer et al., 2010; Somers et al., 2010). In this system, the transfection cassette is flanked by loxP sites, and can be cleaved off by

introducing the Cre-recombinase after successful reprogramming. The Cre-recombination can be achieved by using picornaviral 2A plasmids or adenoviral Cre (Menon et al., 2016). Such a reprogramming construct called STEMCCA is now widely used with reprogramming efficiencies of 0,1-1,5% (Somers et al., 2010). However, although creating transgene-free iPSCs, this system still leaves a genomic scar (LoxP-site), causing possible insertional mutagenesis (Brouwer et al., 2016). The iPSCs generated this way will still be lacking in safety, especially for clinical purposes.

2.2.4.2 Non-integrating viruses

Because of the safety issues related with the use of retroviruses, other methods of generating footprint-free iPSCs have been developed. Non-integrating, viral methods include transfection with adenoviral or sendai-viral vectors. The reprogramming efficiencies using replication-deficient adenoviruses have been very modest, 0,0002% with human cells (Zhou and Freed 2009), and would need a lot more optimisation for it to have useful application in iPSC generation (Malik and Rao, 2013). An F-gene deficient form of the single-stranded, negative-sense RNA sendai virus have been shown to infect a wide range of host cells (Tokusumi et al., 2002), and produces protein in large quantities (Malik and Rao, 2013). The virus replicates in the host cell cytoplasm, which makes it an appealing candidate for reprogramming since it does not integrate to the host cell genome. Moreover, the viral RNA will usually be completely lost at approximately p. 10, creating footprint-free iPSCs. (Malik and Rao, 2013). The viral particles can also be removed by antibody-mediated negative selection against surface protein HN on the virus (Fusaki et al., 2009).

A modified sendai-virus with mutations on polymerase-related genes has been created, and as a result temperature-sensitive viruses that can be removed by a temperature increase are achieved (Brouwer et al., 2016). Traditional methods using four different viruses each containing one of the four reprogramming factors are in use, but a novel system containing KLF4, OCT4 and SOX2 has been developed, and showed an increase in the reprogramming efficiency when used together with a virus containing C-MYC (Fujie et al., 2014). Another sendai-virus method based on the temperature-sensitive variant has been developed, and contains all the four transcription factors in one virus to ensure stoichiometric amounts of all four transcription factors (Nishimura et al., 2011). Human fibroblasts and blood cells have been reprogrammed with efficiencies of 0,1% and 1% (Fusaki et al., 2009; Seki et al., 2010; Ban et al., 2011), comparable to the lentiviral method but producing iPSCs of higher quality.

2.2.4.3 PiggyBac

PiggyBac (PB) is a mobile linear genetic element, a transposon, that can transpose between chromosomal TTAA sites with the help of a transposase. The PB-transposase recognises specific inverted terminal sequences in the transposon, and integrates them between the TTAA sites. Usually the system consists of a donor plasmid comprising the transposon with the transgenes and a helper plasmid expressing the transposase (Gonzalez et al., 2011). After reprogramming, the PB-transposase can be used to cleave out the transposon, leaving no genomic scar unlike the Cre/loxP-system. (Menon et al., 2016) In addition to creating footprint-free iPSCs, the PB-transposon can be used to reprogram any type of cell, and is a completely xeno-free system (Brouwer et al., 2016). Successful reprogramming of human embryonic fibroblasts using the PB containing the Yamanaka-factors resulted in efficiencies of 0,02-0,05% (Kaji et al., 2009). However, the full removal of the transposon has not been demonstrated. Since the PB-transposon is integrated momentarily into the host cell genome, it can integrate into a transcriptional region and hamper the expression of endogenous genes. The human genome also contains endogenous PB-transposase sites, which may respond upon introduction of the PB-transposon. (Brouwer et al., 2016) Moreover, some studies have suggested that removing large copy numbers of the transposon might be difficult. (Menon et al., 2016).

2.2.4.4 Minicircle or plasmid DNA

The reprogramming factors can also be introduced to the host cells as DNA molecules as plasmids or minicircle DNA, usually with electroporation (Han et al., 2015). Compared to the viral gene delivery, these methods are relatively simple and fast, since no laborious production of viral particle is required. In addition, the electroporation process is extremely quick and relatively inexpensive.

The minicircle is a supercoiled small DNA molecule consisting only of a eukaryotic promoter and the expressed cDNA (Malik and Rao, 2013). Unlike traditional plasmids, they have no bacterial backbone and might be less immunogenic (Brouwer et al., 2016). The reprogramming efficiency using minicircle vectors are, however, very low. For example, Narsinh et al were able to reprogram human adipose stromal cells with a modest efficiency of 0.005% (Narsinh et al., 2011). Usually the host cells need to be transfected multiple times to achieve full reprogramming but recently a CoMIP minicircle vector needing only one transfection was constructed by Diecke et al. (Diecke et al., 2015). The construct was able to achieve successful reprogramming, albeit with a very low efficiency.

Another way to achieve reprogramming is by using an episomal plasmid based on the Epstein-Barr Nuclear Antigen-1 (EBNA-1). Usually the plasmids are expressed only transiently, but the oriP-EBNA-1 plasmid allows for a stable expression of reprogramming factors for a longer period. Thus, only one transfection is needed. The oriP-EBNA1-plasmid can attach to the host chromatin, where it is replicated along with the chromosomal DNA once per each cell cycle. Although attached to the chromosomal DNA, the use of an oriP-EBNA1 plasmid is a non-integrating method generating iPSCs. However, as with the minicircle DNA, the efficiencies of iPSCs generation remain low. By transduction of three oriP-EBNA1 plasmids containing the OCT4–SOX2–NANOG–KLF4, OCT4–SOX2–SV40LT–KLF4 and C-MYC–LIN28 genes, fibroblasts were reprogrammed at a very low efficiency (Yu et al., 2009; Schlaeger et al., 2015). The efficiency of the method could be enhanced considerably by suppressing p53 and using a non-transforming L-MYC instead of the oncogenic C-MYC (Okita et al., 2011). A study by Hu et al. also showed that no plasmid was anymore detectable at passage 15, suggesting that the oriP-EBNA1-plasmid will be lost during time (Hu et al., 2011). Regardless of the low efficiencies, the episomal reprogramming has become one of the preferred non-integrating method for generating iPSCs owing to the high quality of the generated iPSCs (Brouwer et al., 2016).

2.2.4.5 RNA delivery

In order to completely avoid the introduction of genetic or other viral material into the host cell, mRNA can be used (Warren et al., 2010). By direct delivery of synthetic mRNA containing the Yamanaka-factors Warren et al. could reprogram human fibroblasts at a high efficiency of 1,4%. The mRNA has to be processed with phosphatase to create capped 5' end, and the ribonucleoside based cytidine and uridine replaced by modified 5-methylcytidine and pseudouridine to reduce immune responses. When also including LIN28, culturing at 5% O_2 and valproic acid, the reprogramming efficiencies reported were as high as 4,4% (Warren et al., 2010). Although efficient and totally footprint-free, this method is very labor-intensive due to short half-lives of mRNA-molecules (Brouwer et al., 2016). Although the half-life can be increased by adding a 5'-guanine cap (Warren et al., 2010), constitutive addition of mRNA has to be conducted for 7 days. Commercial products for reprogramming are, however, available (Malik and Rao 2013).

In addition to mRNA, also miRNAs have been used to achieve successful reprogramming as discussed in chapter 2.2.3. By choosing miRNAs that are strongly expressed in ESCs,

successful reprogramming has been achieved by various groups. The expression of mir302/367 sequences delivered with a lentivirus was able to reprogram commercial human fibroblasts at a high efficiency of even 10% (Anokye-Danso et al., 2011). In another study, human dermal fibroblasts and stromal cells were reprogrammed by transfection of miRNAs mir-200c, mir-302s and mir-369. Reprogramming efficiency was, however, extremely low, 0,002%. As with traditional mRNA delivery, delivering the miRNAs to the cells as such also requires multiple transfections making the process more laborious. (Miyoshi et al., 2011)

2.2.4.6 Protein delivery

One interesting approach of generating iPSCs without viral or other genomic contamination is the introduction of pluripotency factors as proteins into the cells. Proteins can be delivered to cells fused with peptides such as HIV transactivator of transcription or polyarginine (Inoue et al., 2006; Michiue et al., 2005; Wadia and Dowdy, 2002). In a study by Kim et al., human fibroblasts were successfully reprogrammed with poly-arginine-tagged Yamanaka-factor proteins, albeit with a low reprogramming efficiency of 0,001% (Kim et al., 2009). Although plausible, the low efficiency and difficulty and labor-intensity of producing and purifying large amounts of bioactive proteins makes this strategy ill-suited for routine reprogramming (Gonzalez et al., 2011). As with synthetic mRNA, multiple rounds of transfection of protein is needed to maintain high enough levels of transcription factors for reprogramming (Brouwer et al., 2016).

2.2.5 Reprogramming phases

The reprogramming mechanisms still remain somewhat unknown. The first challenge of the early iPSC-research was to define if the cells truly resemble ESCs. This was in fact proven to be true morphologically, functionally, as well as transcriptionally and epigenetically (David and Polo, 2014; Maherali et al., 2007; Mikkelsen et al., 2008; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). The epigenetic differences observed in some iPSC-lines compared to ESC-lines were shown to be caused mainly by the reprogramming method used (Yamanaka 2012), and can be diminished during passaging of the iPSCs (Chin et al., 2009; Nishino et al., 2011; Polo et al., 2010). Based on large transcriptomic studies of fibroblast reprogramming, Samavarchi-Tehrani et al. divided the reprogramming into three distinct phases: initiation, maturation and stabilization (Samavarchi-Tehrani et al., 2010), depicted in **Figure 3**. Each phase consists of typical events and is characterised by specific molecular markers.

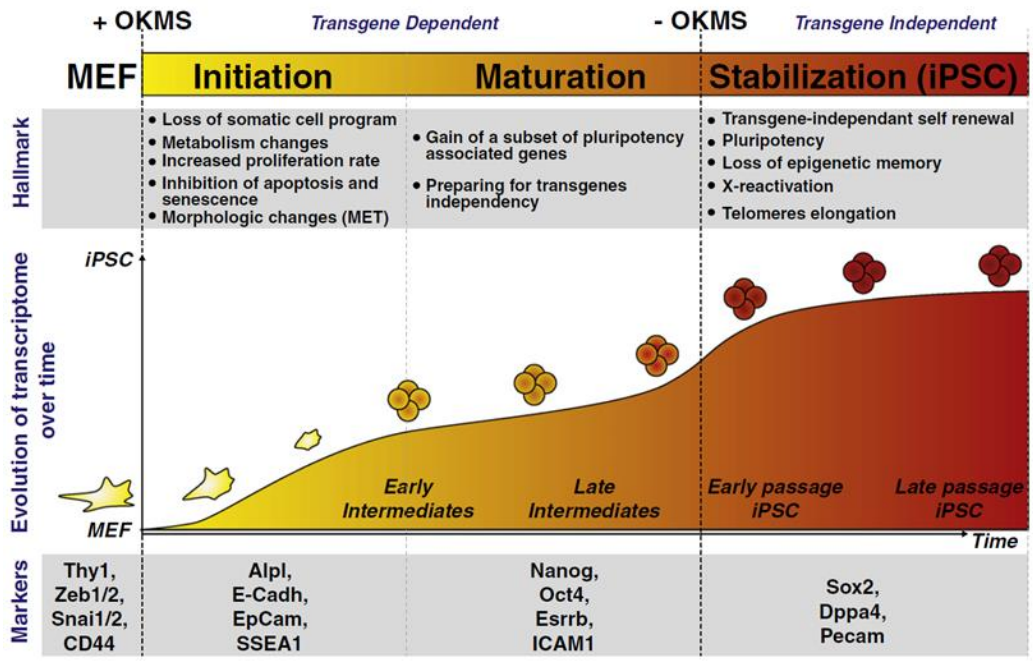

Figure 3. Phases of reprogramming. Reprogramming of iPSCs is thought to consist of three distinct phases: initiation, maturation and stabilisation, all characterised by specific events and markers (David and Polo, 2014)

So far, the initiation phase is the most well-known (David and Polo, 2014). The most used cell type to study the mechanisms are fibroblasts, which in the initiation phase are characterised by a change in the morphology from mesenchymal-to-epithelial transition (MET). Molecular markers for this event includes loss of transcription factors Snai1/2 and Zeb1/2 (David and Polo, 2014; Mikkelsen et al., 2008; Stadtfeld et al., 2008; Samavarchi-Tehrani et al., 2010), and subsequent gain of epithelial markers Cdh, Epcam or the epithelia-associated miRNA-200 family (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Other markers, such as Thy1 and CD44 are lost, and pluripotency markers alkaline phosphatase and stage-specific embryonic antigen (SSEA)-1 gained (O'Malley et al., 2013; Hansson et al., 2012; Polo et al., 2012; Samavarchi-Tehrani et al., 2010; Brambrink et al., 2008; Mikkelsen et al., 2008; Stadtfeld et al., 2008). Also, two kinases have been identified as likely barriers of reprogramming: Tesk1 and LIMK2. When TESK1 was inhibited with a siRNA, the reprogramming efficiency was significantly improved (Sakurai et al., 2014).

In addition to the MET-associated events, also the acquisition of ESC-like properties including proliferation and resistance to apoptosis or cell senescence are important features taking place during the initiation phase (David and Polo, 2014; Marion et al., 2009a; Utikal et al., 2009b; Mikkelsen et al., 2008). Interestingly, although most the cells have been shown to be able to

initiate reprogramming, only a small portion of these cells can undergo full reprogramming (Polo et al., 2012). The mechanism is not known, but one proposed theory is the innate immunity, that would trigger protein degradation in the reprogramming-refractive cells (David and Polo, 2014).

The changes in gene and protein expression during the initiation phase suggests a hierarchical network of events caused by the interaction of the transcription factors, co-factors and the chromatin. One mechanism of how this happens was proposed by Soufi et al.: the high concentration of the transcription factors can bind to more genes than they would with physiological concentrations, thus inducing reprogramming (Soufi et al., 2012). The study also suggests that the transcription factors Oct4, Sox2 and Klf4 bind to inactive DNA regions, while Myc only binds to accessible regions, serving as a transcriptional response amplifier in the activated DNA regions (Lin et al., 2012; Nie et al., 2012). Myc is thought to be responsible for the induction of MET, while the other transcription factors mostly serve as pioneers (Soufi et al., 2012; Sridharan et al., 2009) At the chromatin level changes occur only as histone modifications, but not as epigenetic changes (David and Polo, 2014; Polo et al., 2012).

The transition from the initiation to the maturation phase is considered as the major bottleneck phase of reprogramming (David and Polo, 2014). The maturation phase is characterised by the activation of the first pluripotency genes (Polo et al., 2012; Samavarchi-Tehrani et al, 2010). The first markers that can be detected during this phase include, Fbxo 15, Sall4 and endogenous Oct4. After this, also Nanog and Esrrb can be detected. Fbxo15 alone is however a poor marker, since it has also been shown to be active in only partially reprogrammed cells (David and Polo, 2014; Takahashi and Yamanaka, 2006). More reliable markers used since have been Nanog and Oct4 (Maherali et al., 2007; Okita et al., 2007), although notable that none of these factors alone is either a guarantee of complete programming (Buganim et al., 2012). At the verge of entering the stabilisation phase, factors such as Sox2 and Dppa4 can be detected (Buganim et al., 2012; Polo et al., 2012; Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008). The acquisition of pluripotency markers is thought to happen in a sequential way with some markers expressed earlier in the maturation phase, and others first late in the stabilisation phase (Polo et al., 2012; Buganim et al., 2012).

The stabilisation phase includes the changes that happens in the iPSCs after becoming pluripotent (Ho et al., 2011). In this phase the cells acquire the full pluripotency signature, and

in the end a pluripotent state that is maintained without the help of ectopic expression of the reprogramming factors (Okita et al., 2007; Wernig et al., 2007). The characterisation of iPSCs is done during this phase (David and Polo, 2014). In mouse iPSCs, the inactivated X chromosome is rendered active again during the stabilization phase (Stadtfeld et al., 2008). The phase is also characterised by many epigenetic changes, many of which remain poorly known. One important notion in mouse cells is the elongation of telomeres into an embryonic level (Stadtfeld et al., 2008; Marion et al., 2009b). During extended periods in culture, the iPSCs also become epigenetically more like ESCs (Nishino et al., 2011; Chin et al., 2009; Polo et al., 2010), while at the same time losing the epigenetic memory of the donor cell type. The epigenetic resetting can also be enhanced using 5-aza-deoxycytidine (AZA). (Kim et al., 2011; Ohi et al., 2011; Polo et al., 2010). At least one DNA methylation factor, AID, has been shown to be involved in the epigenetic reset (Bhutani et al., 2010; Kumar et al., 2013), but possibly also the TET-family and DMNTs play a role in this event (Polo et al., 2012). Further studies to unveil the mechanisms underlying the epigenetic remodeling are, however, required (David and Polo, 2014).

2.2.6 Characterisation of induced pluripotent stem cells

To assess the quality of the generated iPSCs, they must be characterised on many different levels. These levels and methods to study them are presented in **Figure 4**. As the first sign of iPSC formation is the typical morphology: the PSC morphology is defined by compact colonies with defined borders, having small cells with a high nucleus to cytoplasm ratio and large nucleoli (Thomson et al., 1998). For feeder-free monolayer cultures, the morphology is less defined (Brouwer et al., 2016). In addition to the typical morphology, iPSCs proliferate extensively in cell culture (Thomson et al., 1998).

In addition to morphological characterisation, many cellular and molecular level assays are used to characterise the cells (Brouwer et al., 2016). iPSCs are fully reprogrammed only when the transgenes are silenced and endogenic pluripotency genes turned on (Hotta and Ellis, 2008). Thus, the silencing of transgenes needs to be confirmed. The expression of various pluripotency markers (presented in **Figure 4**) is assessed by RT-PCR at mRNA level and at protein level by immunocytochemistry. The presence of one marker is not necessarily an indication of complete reprogramming (Buganim et al., 2012), and usually many of these markers are used. Since pluripotent stem cells are also characterised by a high enzymatic activity of phosphatases, an alkaline phosphatase assay is often performed (Brouwer et al., 2016).

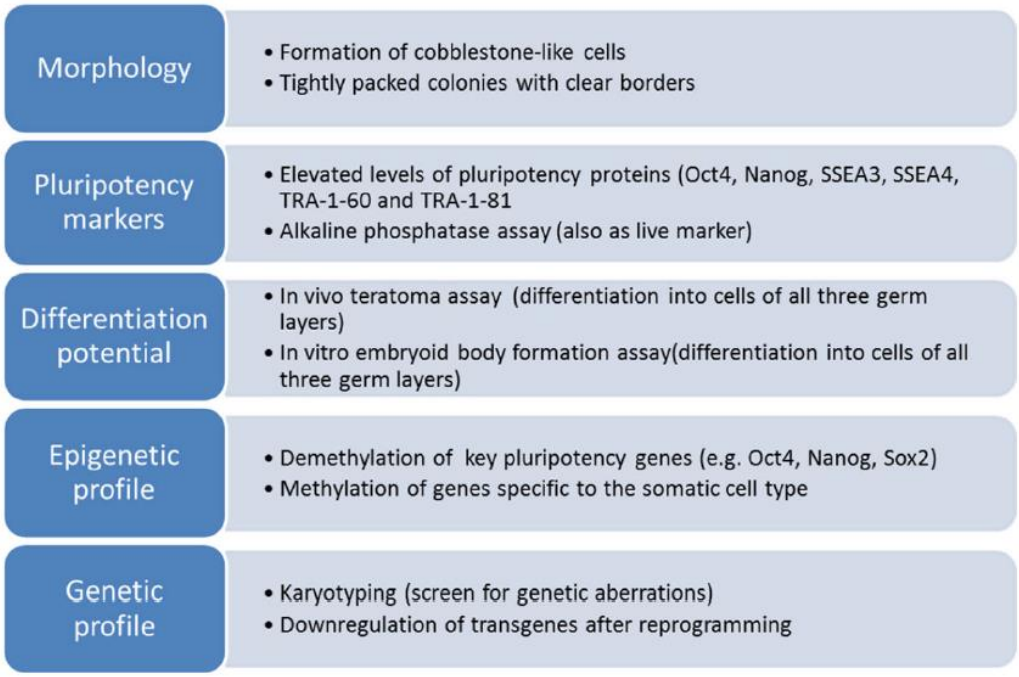

Figure 4. Characterisation of iPS-cells. To verify the pluripotency and full reprogramming of the generated iPS-cells, many different methods can be used. While it's not necessary to perform all methods, no method alone can confirm good quality of the iPSCs. (Brouwer et al., 2016)

The differentiation potential of the iPSCs is usually assessed *in vitro* and *in vivo*. The pluripotent stem cells should be able to differentiate into all three germ layers. An *in vitro* assay of the differentiation potential includes an embryoid body (EB) assay, usually performed in floating culture (Yu et al., 2007; Takahashi and Yamanaka 2006; Itskovitz-Eldor et al., 2000). *In vivo* – differentiation potential is usually assessed by a teratoma formation assay usually performed by injection of cells into immunodeficient mice (Thomson et al., 1998). The detection of the different germ layer can be subsequently verified by RT-PCR of germ-layer specific genes.

Since genetic and epigenetic changes can occur during the generation of iPS-cells (Doi et al., 2009; Kim et al., 2010; Polo et al., 2010), the genetic and epigenetic profiles of the iPSCs should also be studied. Large chromosomal aberrations can be detected with a karyotyping analysis. Since DNA methylation is an indicator of gene silencing, the methylation states of the stem cell specific –endogenes and donor-cell type –specific genes can be assessed. For example, NANOG and OCT4 are unmethylated during reprogramming, which indicates their active transcription (Mikkelsen et al., 2008). During reprogramming, the somatic donor cell -specific genes should also be silenced, indicated by methylation. At the same time, pluripotency genes should be activated, indicated by demethylation. (Brouwer et al., 2016)

While many different assays can be used to characterise the created iPSCs, no method alone is sufficient to confirm good quality of the iPSCs. Thus, a combination of methods should be used. (Brouwer et al., 2016)

2.3 Cardiomyocyte differentiation

The generation of cardiomyocytes from iPSCs is of interest for multiple reasons. Since cardiac development cannot be studied in the developing embryos, the cardiogenesis can be studied *in vitro* with the help of iPSCs. Moreover, these cardiac *in vitro* -models can also be useful in basic research of cardiac function such as electrophysiology or protein chemistry. Genetic cardiac disorders can be studied by the generation of patient- and disease-specific iPSC-lines from patients with these disorders. Moreover, these models can be used for drug and toxicity screening of different compounds. In the more distant future, iPSC-derived cardiomyocytes can also be used in regeneration and cell therapies, such as repair of the human heart after a myocardial infarct. (Mummery et al., 2012)

During extended periods of culture, the cells gain a more mature phenotype described by the loss of proliferative ability, elongation, subtype specific action potential profile, changes in gene expression and an increased beat frequency. Even so, the cells resemble more fetal than adult cardiomyocytes. (Batalov and Feinberg, 2015) To develop better-quality cardiomyocytes for research and therapeutic purposes, the maturation process and factors involved need to be studied. In addition, methods to apply these to iPSCs-derived cardiomyocytes also have to be developed.

2.3.1 Differentiation methods

The cardiomyocyte differentiation protocols for iPS-cells were first established for ES-cells, and later adapted to iPSCs (Batalov and Feinberg, 2015). Currently, differentiation methods can be divided into three categories: 1) co-culture with mouse endoderm-like (END-2) stromal cells 2) differentiation in EBs in suspension culture and 3) 2D monolayer differentiation (Batalov and Feinberg, 2015). All methods however, produce cardiomyocytes with an immature phenotype when compared to adult cardiomyocytes. The cells can be matured further by various methods. These methods include prolonged time in culture (for even longer than a year), electromechanical stimulation, treatment with tri-iodo-L-tyronine, transgenic expression of cardiac-specific proteins or by co-culturing them with non-cardiomyocytes. (Batalov and Feinberg, 2015).

2.3.1.1 Co-culture with END-2 cells

The cardioinductive signals during embryonic development arise likely from a direct cell-cell contact or by factors secreted from the embryonic endoderm (Rajala et al., 2011). The END-2 cells from mouse P19 embryonal carcinoma are used to mimic the embryonal endoderm, and to drive the differentiation into cardiomyocytes. The differentiation efficiency is usually fairly low, but can be improved by the use of AA (Passier et al., 2005; Takahashi et al., 2003), or in the absence of serum (Rajala et al., 2011). With the use of a p38 MAPK inhibitor, an efficiency as high as 25% could be achieved (Graichen et al., 2008). Factors identified from END-2 that enhance the cardiomyocyte differentiation, can also be used to further enhance the differentiation efficiency (Rajala et al., 2011). Advantages of the END-2 differentiation method are its inexpensiveness and simplicity (Batalov and Feinberg, 2015).

2.3.1.2 Embryoid body differentiation

The EB differentiation method is a method that mimics the early embryonic development (Batalov and Feinberg, 2015). It relies on either spontaneous differentiation or a combination of physical and chemical factors to direct the differentiation of iPSCs into cardiomyocytes. The spontaneous differentiation is performed in suspension culture, where the iPSCs aggregate to form the EBs and spontaneously differentiate into a myriad of cell types. Inside the formed EBs, contracting areas with functional properties of cardiomyocytes are found, and can be isolated and re-plated for further differentiation. The efficiency obtained by this method is, however, low with under 10% of the cells differentiating into cardiomyocytes. (Rajala et al., 2011) Spontaneously formed EB-aggregates vary in size and morphology. The variation between the EBs can, however, be decreased by hanging-drop and forced-aggregation methods (Yoon et al., 2006). The differentiation towards cardiomyocytes can be further enhanced by the addition of growth factors, morphogenes or by transgenic modifications (Rajala et al., 2011). At least with ES-cells, the efficiency has been shown to significantly increase by the addition of 5-AZA (Yoon et al, 2006). In addition, low oxygen tension using a 4% O_2 level rather than the atmospheric 20% yielded a higher amount of cardiomyocyte differentiation (Niebruegge et al., 2009). Also electrical stimulation has been applied resulting in increased differentiation efficiency (Serena et al., 2009).

2.3.1.3 2D monolayer culture

The 2D monolayer differentiation method is based on guidance by small molecules and growth factors added to the culture medium. In comparison to the EB and END-2 methods, the 2D monolayer culture method also results in more mature cardiomyocytes. Also cardiomyocytes

showing signs of subtype specification have been created by this method with cardiomyocyte yields as high as 85- 95%. (Batalov and Feinberg, 2015) The first paper reporting monoculture differentiation using hESCs was published in 2007 (Laflamme et al., 2007). At first, a confluent monolayer was cultured on Matrigel in MEF-conditioned medium. After this, the medium was changed into a chemically defined RPMI-B27 medium complemented with Activin-A, bone morphogenetic protein (BMP)-4 at precise time points, followed by culture in pure RPMI-27 for two weeks. With this method, over 30% of the cells differentiated into spontaneously beating cardiomyocytes. However, a lot of variation existed between different cell lines. In 2012 Lian et al. could improve the monolayer culturing method by stimulation of Wnt (a mammalian ortholog of the Wingless gene observed in *Drosopohila*) /β-signaling with the addition a GSK3-inhibitor at the beginning of differentiation. They also noted that insulin in the B27 medium supplement serves as an inhibitor for cardiomyocyte differentiation. The adding of GSK3-inhibitor and removal of insulin led to both increased consistency between lines and an increased differentiation efficiency of 82-95%. (Lian et al., 2012) Since the B27 medium contains factors with not yet fully defined effects on the differentiating cells, media containing of only a few known components have been tested lately. For example, E8 media also used with iPSCs has proven efficient, as well as a CDM3 medium containing only 3 components. These media also decrease the costs of differentiation. (Batalov and Feinberg, 2015)

2.3.2 Cardiogenesis and iPSC-differentiation mechanisms

One of the first events in embryonic development is the formation of the heart (Rajala et al., 2011). Heart development requires precise migration, proliferation and differentiation of many cell types originating from different embryonic origins. These processes need to be tightly orchestrated in a timely manner by different molecular pathways. (Roche et al., 2013) Studies with mice and chick embryos have shown that the heart tissue is formed from three major mesoderm-originated lineages including the cardiac myocyte, the vascular smooth muscle, and the endothelial cell lineages. (Rajala et al., 2011) Early in gastrulation, the cardiac progenitor cells arise from the anterior lateral mesoderm and migrate through the primitive streak. These early progenitor cells are comprised of a cell population called the cardiac crescent. Positive and negative signals from the underlying endoderm are responsible for inducing cardiac specification of the cardiac crescent. One of the earliest markers for cardiac specification is Wnt. (Roche et al., 2013) The progenitors that form the heart fields coalesce and form two parallel vessels, which are in turn fused to form the cardiac tube. After rightward looping and

a series of septation and fusion events, the four-chambered heart forms and matures further before birth. It is first after birth that the cardiomyocytes undergo terminal differentiation and lose their ability to proliferate. (Roche et al., 2013) The events leading to the generation of the heart are controlled by many transcription factors. (For a more detailed review of the factors and their role in cardiogenesis, see Roche et al., 2013)

The differentiation of iPSCs into cardiomyocytes *in vitro* mimics the cardiogenesis observed in the embryo. The well-orchestrated cardiac development includes the expression of multiple signal transduction proteins and transcription factors, the most studied of which are Wnts/Nodal, BMPs and FGFs (Rajala et al., 2011). In addition to the right factors, also their timely manner is of importance, and certain factors can serve as inhibitors during a certain period of time, and as activators at another time point. Thus, the timing of their addition to guide the differentiation of iPSCs is of crucial importance. Cardiomyocytes can be differentiated in four steps: 1) formation of mesoderm, 2) the patterning of mesoderm toward anterior mesoderm or cardiogenic mesoderm, 3) formation of the cardiac mesoderm and 4) maturation of early cardiomyocytes (Rajala et al., 2011). The steps and typical markers observed during those steps are outlined in **Figure 5**.

The first step has been well characterised, and many studies show that Wnts, BMPs and transforming growth factor (TGF) β- family member Nodal (or Activin A) are important in inducing mesoderm. The two latter steps are less well defined for human iPSCs. However, studies with chick and xenopus embryos suggest that Nodal and Wnt inhibition plays a role in cardiomyocyte formation. Thus, Dickkopf-1, a Wnt antagonist, is usually used in differentiation protocols. Another important signal pathway is one mediated by a transmembrane receptor called Notch. It induces the expression of many growth factors including Wnt5a, BMP6, and Sfrp1 that in turn increase the number of cardiac progenitors. The last step, where committed cardiac progenitors mature into beating cardiomyocytes, usually occurs spontaneously *in vitro*. (Rajala et al.,2011)

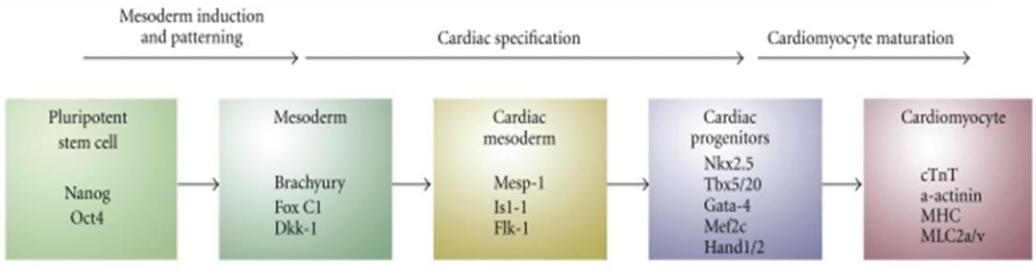

Figure 5. The steps in cardiac differentiation of iPSCs and typical markers expressed during those steps. (Rajala et al., 2011)

In addition to the various growth factors and transcription factors, miRNAs have been shown to affect the differentiation process. miR-1, miR-133, miR-206, and miR-208 are expressed in the heart (Callis et al., 2008; van Rooji et al., 2008), while miR-143 and miR-145 are also thought to have an important role in cardiomyogenesis. (Wang et al., 2008). For example, miR-1 was able to increase the amount of beating aggregates in EB differentiation. MiR-133 also promotes the differentiation of the early mesoderm, but after that serves as an inhibitor of cardiac commitment. (Ivey et al., 2008) Better knowledge of the later steps in cardiomyocyte differentiation could help in identifying factors that could increase differentiation efficiency and further enhance cardiomyocyte maturation.

2.3.3 Characterisation of cardiomyocytes

To characterise the generated cardiomyocytes, the expression of specific molecular markers, structure and functionality can be studied (Rajala et al., 2011). The first sign of cardiomyocyte differentiation *in vitro* is the appearance of spontaneously beating cells (Mummery et al, 2003). The cardiomyocytes originate from a mesodermal origin, and as an early marker of the mesoderm and cardiac lineage differentiation, Brachury T is generally used (Kispert and Herrmann, 1994). Other early markers of cardiomyocyte differentiation are the upregulated gene expression of transcription factors Islet-1, mesoderm posterior 1 and 2, GATA4-binding protein and T-box transcription factor 6 (Graichen et al., 2008; Yang et al., 2008; Brand, 2003). The expression of these genes can be studied at mRNA level by RT-PCR.

Also several structural proteins can be used as markers of cardiomyocyte differentiation, and stained by immunocytochemistry. Markers of a more mature phenotype include cardiac Troponin T, cardiac Troponin I, cardiac α-actinin, desmin, tropomyosin and atrial- and ventricular myosin light chains (Kehat et al., 2001; Mummery et al, 2003; Rajala et al., 2011). In addition, connexin proteins present in gap junctions, such as connexin 43, 40 and 45 can be used as markers (Gaborit et al. 2007), as well as ion channel markers hERG and KCQN1. Also natriuretic peptide can be used as a marker of cardiomyocyte differentiation (Rajala et al., 2011).

In addition to visual and biochemical characterisation, the functionality of the differentiated cardiomyocytes can be characterised by studying the electrophysiology of the cells. Action potentials of single cardiomyocytes can be studied with patch-clamp analysis, and immature iPSC-derived cardiomyocytes are characterised by a slower action potential upstroke and a

relatively depolarised maximum diastolic potential (Rajala et al., 2011). The cardiomyocytes also express altered Ca2+ handling (Batalov and Feinberg, 2015), that can be studied with microelectrode array (MEA).

Altogether, the cardiomyocytes differentiated from iPSCs all express an immature phenotype when compared to adult cardiomyocytes. The more fetal-like phenotype expressed is characterised by a smaller length-to-width aspect ratio, mononuclearity, poor sarcomere organisation and fewer mitochondria when compared to adult cardiomyocytes. (Robertson et al., 2013)

3. Research objectives

The first aim of this study was to establish and characterise new iPSC-lines generated either by an episomal or a sendai-viral method and cultured in traditional conditions including mouse embryonic feeders and KSR-medium supplemented with b-FGF. A comparative view was maintained between the sendai-virally and episomally derived lines from the same patients. To assess the effect of the generation method on cardiomyocyte differentiation efficiency, two of these established and characterised lines from the same patient were differentiated into cardiomyocytes by an END-2 co-culture system for comparison.

Traditional culturing of iPSCs includes undefined and xenogenic components, that pose issues especially for generating GMP-standardised methods for iPSC-generation and maintenance, as well as for possible clinical use of the generated iPS-cells. The other aim of this study was to establish and maintain iPSCs in feeder-free conditions on a Geltrex®-matrix in mTeSR1 medium. Comparison of the reprogramming efficiencies between all episomally derived lines (both MEF-cultured, and the Geltrex®-lines) was also performed.

The last aim was to study the activation of pluripotency genes by real-time qPCR from mRNA samples collected from an earlier and later passages. As the activation of endogenous pluripotency genes is thought to be a slow process (David and Polo, 2014), the genes were studied to see at which passage they are upregulated. Comparison was also done to study the differential expression of the pluripotency genes between patients.

4. Materials and Methods

4.1 Cell lines

All cell lines used in this study were newly reprogrammed from somatic patient cells. The cells used for reprogramming were dermal fibroblasts isolated and cultured from skin biopsies from patients with cardiac diseases. The fibroblasts were reprogrammed either with a Sendai-viral method or an electroporation method, and cultured on mouse embryonic feeders (MEFs) or a cell-free Geltrex®-matrix. Lines used for this study were S1M, S2M, S3M, E1.1M-E1.4M, E2.1M-E2.4M, E3.1M-E3.2M, E4G and E5G. The first letter of the cell line refers to the reprogramming method (sendai virus=S and electroporation= E, respectively) and the latter for the culturing method (MEFs=M and feeder-free Geltrex®=G). Cell line numbers are patient specific, and all in all lines from five different patients were studied. The studied sendai-lines (S1M-S3M) were obtained from the lab technician.

4.2 Reprogramming of patient fibroblasts

Initial reprogramming for electroporation lines E1.4M-E3.2M was performed by PhDs Leena Viiri and Stephano Manzini. 600 000 fibroblasts (500 000 for lines E4G and E5G) were transfected with integration free, episomal plasmid vectors pCXLE-hOCT3/4-shp53-F; pCXLE-hSK; pCXLE-hUL and pCXWB-EBNA1 (Addgene) containing pluripotency genes L-MYC, OCT3/4, SOX2 and KLF4 (plasmid constructs presented in **Appendix A**, Okita et al. 2011). For transfection, the plasmids were used in equimolar amounts (1:1:1:1), i.e. 3 ug of each plasmid, except, 1,7ug of the smaller plasmid pCXWB-EBNA1. Transfection was conducted with the 4D-Nucleofector™ System (Lonza) according to manufacturer's instructions.

Transfected cells were cultured overnight in medium containing Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco by Life Technologies) and 1% L-glutamine (Gibco by Life Technologies). Medium was changed the next day using same basic fibroblast medium supplemented with 1% Pen-Strep (Sigma Aldrich). Cells were incubated for 1 week at +37°C and 5% CO_2. After this cells were trypsinised (Lonza), counted and re-plated at a density of 200 000/well on sterile 6-well plates (Greiner CELLSTAR®, Sigma-Aldrich). Transfected cells co-cultured with mouse embryonic feeder cells were plated and maintained on plates containing feeder cells (as describer in chapter

4.3.1) and feeder-free cultures were maintained on Geltrex® (ThermoFisher Scientific)-coated plates (as describer in chapter 4.3.2). Cells were cultured at +37°C and 5% CO_2 until colony picking.

Sendai-lines S1M-S3M were reprogrammed by the lab technician. 150 000 fibroblasts were transduced with the CytoTune®-iPS Sendai Reprogramming Kit (Invitrogen) containing F-gene deficient Sendai-viruses expressing the four Yamanaka factors. The reprogramming was conducted according to manufacturer's instructions with a MOI of 1.25. After transduction, the sendai-lines were treated similarly to electroporated MEF-grown lines.

3–4 weeks after the transfection/transduction individual iPSC-colonies had formed and were picked onto 24-well plates before being transferred onto 6-well plates one week later. New iPSC lines were established from the colonies that survived after picking, and maintained as described in chapter 4.3.1.

4.3 Maintenance of induced pluripotent stem cells

4.3.1 iPS-cell co-culture with mouse embryonic feeder cells

iPS-cells were cultured on sterile 6-well plates (Greiner CELLSTAR®, Sigma-Aldrich) at +37°C and 5% CO_2 in 3 ml of KSR-medium ((KnockOut™ Dulbecco's Modified Eagle Medium (KO-DMEM); Gibco by Life Technologies) supplemented with 20% KnockOut™ Serum Replacement (KO-SR; Gibco by Life Technologies), 2 mM non-essential amino acids (100x MEM NEAA; Gibco by Life Technologies), 1% L-glutamine (Sigma Aldrich), 1% Penicillin Streptomycin (Pen-Strep; Sigma Aldrich), 0.1 mM β-mercaptoethanol (β-ME; Sigma Aldrich) and 4 ng/ml of β-FGF (Peprotech)) per well. Half of the medium was changed every 2–3 days. To confirm the undifferentiated status, the cells were visualised with light microscopy at least every other day.

Cells were passaged once a week. One day prior to passaging, wells on a standard 6-well plate were coated with 0,1% gelatin (Type A from porcine skin, Sigma) and incubated for 1 hour at +37°C. Excess gelatin was removed and inactivated MEF cells (Mitomycin-C treated, Applied StemCell, Inc.) seeded at a density of 250 000 cells/well. Feeder cells were cultured overnight at +37°C and 5% CO_2 in 3 ml of fibroblast medium containing DMEM supplemented with 10% FBS (Gibco by Life Technologies), 1% L-glutamine (Gibco by Life Technologies) and 1% Pen-

Strep (Sigma Aldrich). The attachment of the feeder cell layer was confirmed with light microscopy before plating of the iPS-cells.

The passaging of the iPS-cells was done by first removing the old feeder cell layer by scraping under the light microscope with a pipette tip. The intact iPS-cell colonies were then treated with 1 ml of KSR-medium supplemented with 1 mg/ml collagenase IV (Gibco by Life Technologies) per well for 4-5 min at +37°C. After incubation, colonies were scraped into fresh KSR-medium, pipetted gently up-and-down a few times and part of the cell suspension was transferred onto new 6-well plates coated with a feeder layer. Cell attachment was confirmed the next day with light microscopy.

Throughout the experiment, stocks of lines were made by freezing colonies in freezing medium containing 10% dimethyl sulfoxide (DMSO; Sigma Aldrich) and 90% FBS (Gibco by Life Technologies). After being frozen down in isopropanol chambers at -70°C, the cells were stored in liquid nitrogen.

4.3.2 iPS-cell culture on feeder-free substrate

iPS-cells cultured on feeder-free Geltrex® substrates were cultured on similar 6-well plated as MEF-cultured cells. 3 ml of mTeSR™1 basal medium (STEMCELL™ Technologies) supplemented with 10% of 5x mTesR supplement (STEMCELL™ Technologies) and 0,5% Pen-Strep was used per well. Half of the medium was changed every 2-3 days and cells inspected visually with light microscopy.

The iPS-cells were passaged every 3–5 days. Prior to passaging, wells on a new 6-well plate were coated with 1:100 Geltrex® in KO-DMEM (Gibco by Life Technologies) for 1 h–overnight at +37°C. Passaging was done by treating the iPS-cells with 1 ml Versene® (Lonza) per well for 2–5 minutes on a +37°C heating plate, after the removal of differentiated cells. When the cells were detaching from the substrate (inspected with light microscopy), Versene® was removed and cells scraped into fresh culture medium. Part of the cell suspension was transferred onto new Geltrex®-coated wells. Cell attachment was confirmed with light microscopy the next day.

Throughout the experiment, stocks of iPSCs were made by freezing colonies in freezing medium (described in chapter 4.3.1). After being frozen down in isopropanol chambers at -80°C, the cells were stored in liquid nitrogen.

4.4 Characterisation of induced pluripotent stem cell lines

4.4.1 Immunocytochemistry

Immunocytochemistry for line E1M-E3M was performed at p. 10 and for S1M-S3M at passages 10,12 and 13, respectively. Immunocytochemistry for line E4G was performed at p. 19.

Initial washing was done with 1X phosphate-buffered saline (PBS) before fixing for 20 min with 4% paraformaldehyde (PFA; Sigma Aldrich) in 1X PBS. After fixing, the cells were either stored in 1X PBS at +4°C or stained immediately. Permeabilisation and blocking was performed at the same time by 10% normal donkey serum (NDS; Millipore), 0,1% Triton-X 100 (Sigma Aldrich) and 1% bovine serum albumin (BSA; Sigma Aldrich) in 1X PBS for 45 min. After blocking, cells were washed once with 1X PBS containing 1% BSA, 1% NDS and 0,1% Triton-X 100. Incubation with primary antibodies (diluted in previous washing solution) included pluripotency markers Sox2 (Sox2 goat IgG; Santa Cruz), Tra 1-60 (Anti-Tra 1–60 mouse IgM; Millipore) and Tra 1-81 (Anti-Tra 1–81 mouse IgM; Millipore) all diluted 1:200; SSEA-4 (SSEA4 mouse IgG; Santa Cruz) diluted 1:100 and Oct3/4 (Anti-human Oct3/4 goat IgG; R&D Systems, Inc.) diluted 1:400. Primary antibodies were incubated overnight at +4°C.

After overnight incubation with primary antibodies, cells were washed three times with 1% BSA in 1X PBS, and incubated light-protected with secondary antibodies in 1% BSA in 1X PBS for 1 h. The secondary antibodies used at a dilution of 1:800 were Alexa Fluor 568 nm donkey anti-goat IgG (Invitrogen) for Oct-3/4 and Sox-2; Alexa Fluor 568 nm goat anti-mouse IgG H&L (Invitrogen) for SSEA-4 and Alexa Fluor 568 nm goat anti-mouse IgM M chain (Invitrogen) for TRA 1-60 and TRA 1-81. Following incubation, cells were washed twice with 1X PBS and phosphate buffer (PB) before mounting with Vectashield Mounting Medium including 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

In addition to the pluripotency characterisation, immunocytochemistry for Geltrex®-cultured cell line E4G at p. 7 and p. 17 was performed similarly with early neural marker primary antibodies Nestin (Anti-Nestin mouse IgG; Millipore) and microtubule-associated protein (MAP)-2 (Anti-MAP-2 rabbit IgG; Millipore) at dilutions 1:1000 and 1:400, respectively. Secondary antibodies used at a dilution of 1:800 were Alexa Fluor 568 nm goat anti-mouse IgG H&L and Alexa Fluor 568 nm goat anti-rabbit IgG, respectively. The samples were stored light-protected at +4ºC and visualised with Olympus IX51 fluorescence microscope. Images were acquired using Olympus DP30BW and edited with DP manager software (Olympus).

4.4.2 Expression of pluripotency markers

4.4.2.1. RNA sample collection, extraction and cDNA synthesis

All RNA samples were collected using 600 µl of RLT buffer (QIAGEN) containing 10 µl of β-ME (Sigma Aldrich) per 1 ml of lysis buffer. Before sample collection from feeder-cultured iPS-cells, MEF-cells were scraped out into the growth medium under the microscope using a pipette tip, leaving the iPS-cell colonies intact. After scraping the growth medium was removed and cells were washed twice with 3 ml of 1X PBS. For feeder-free cultures on Geltrex®, the growth medium was removed and cells washed twice with 3 ml of 1X PBS. After washing the RLT buffer containing β-ME was added to lyse the cells. Collected samples were stored at -80°C until RNA extraction.

RNA extraction was performed using the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. Additional on-column DNase digestion was performed to digest remaining DNA. The purified RNA samples were eluted in 30 µl of sterile H_2O. Concentrations and purities of the eluted RNA samples were measured spectrophotometrically (NanoDrop Spectrophotometer ND-1000). Extracted RNA samples were stored at -80°C.

RNA samples were reverse transcribed into cDNA by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Reaction master mix contained 10X RT buffer, 10X RT random primers, 25X dNTP mix (100 mM), Multiscribe™ Reverse Transcriptase, RNase inhibitor (Riboblock RNase Inhibitor, Thermo Scientific) and nuclease-free H_2O in a volume of 10 µl per reaction. A total of 500 ng of extracted RNA was used for each reaction, in a volume of 10 µl. Negative –RT controls for all samples were also prepared by replacing the Multiscribe™ Reverse Transcriptase with nuclease-free H_2O, as well as a negative H_2O control replacing the RNA in the reaction. The cDNA-transcription was carried out with the Thermal Cycler Mastercycler (Eppendorf) and reaction conditions were 1) 25°C, 10 min; 2) 37°C, 120 min; 3) 85°C, 5 min and 4) 4°C until storage. Synthesised cDNA was stored at -20ºC.

4.4.2.2 EXO-PCR

To verify that exogenic plasmid material is no longer present in the iPS-cells, DNA samples from episomally transfected cell lines were collected at passage 8. Cells were washed twice with 1X PBS before lysis in 80 µl of T1 buffer (Macherey-Nagel). The T1 buffer-lysed samples were stored at -70°C until DNA extraction. DNA extraction was performed using Nucleospin®

Tissue XS –kit (Macherey-Nagel) according to manufacturer's instructions. Instead of an elution volume of 20 µl suggested in the protocol, a volume of 30 µl of Buffer BE was used. Extracted DNA was stored either at -20ºC or -70°C, and the DNA concentrations and purities were measured spectrophotometrically (NanoDrop Spectrophotometer ND-1000). Before exogenic PCR, all DNA samples were diluted to a final concentration of 25 ng/µl.

The absence of exogenic plasmid DNA in the electroporated cell lines was assessed by PCR of the EBNA-1 gene, which is present in all the transfected plasmids. A pCXWB-EBNA1 plasmid dilution series with concentrations of 1 ng/µl–1 fg/µl was used as the positive control and H_2O as a negative control. Master mixes for each reaction contained 2 µl of Dynazyme buffer, 0.2 µl of Dynazyme polymerase II (Thermo Scientific), 0.4 µl of 10mM dNTP mix (Fermentas), 0.4 µl of 25 mM MgCl (Fermentas), 12 µl of RNAse free H_2O, 2 µl of both 5 µM EBNA-1 primers and 1 µl of sample. PCR conditions were 1) 94°C, 2 min; 2) hybridization at 94°C, 30s; 3) annealing at 60°C, 30s; 4) elongation at 72°C, 30s; and 5) final elongation at 72°C, 5 min; 6) storing at 4°C until gel electrophoresis. Steps 2–4 were repeated 40X. All genes, primers and their annealing temperatures are presented in **Table 2**.

Exogenic PCR for Sendai-lines S1M-S3M was done from RNA samples extracted as described in chapter 4.4.3. Since cells should be free of viral material at p. 10 (Griesenbach et al., 2005), exo-PCR was conducted at later passages 14 and 15. As a positive control, cDNA from an early passage (p. 3) of a sendai-transfected line was used. Master mixes were prepared similarly as for electroporation lines except for adding 1 µl of DMSO and 50 ng of cDNA per reaction. PCR conditions were 1) 94°C, 2 min; 2) hybridisation at 94°C, 30s; 3) annealing at 55°C, 30s; 4) elongation at 72°C, 30s; 5) final elongation at 72°C, 5 min; 6) storing at 4°C until gel electrophoresis. Steps 2–4 were repeated 40X. All genes, primers and their annealing temperatures are presented in **Table 2**.

4.4.2.3 ENDO-RT-PCR

The expression of endogenic pluripotency genes was studied with endo-RT-PCR. RNA sample collection was performed from same or closely following passages as for exo-PCR and reverse transcribed as described in chapter 4.4.3. Master mixes for endogenic genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (endogenic control), reduced expression (REX)-1, OCT3/4 and C-MYC consisted of 2 µl of Dynazyme buffer and 0,2 µl of Dynazyme polymerase II (Thermo Scientific), 0,4 µl of 25 mM MgCl (Fermentas), 0,4 µl of 10mM dNTP mix

(Fermentas), 2 µl of both 5 µM primers, with 30 ng of cDNA per reaction, adding RNAse-free water to a final volume of 20 µl per reaction. For NANOG and SOX2 1 ul of DMSO was added per reaction. PCR conditions were 1) 94°C, 2 min; 2) hybridisation at 94°C, 30s; 3) annealing at X°C 30s; 4) elongation at 72°C, 30s; and 5) final elongation at 72°C, 5 min; 6) storing at 4°C until gel electrophoresis, which was performed as described in chapter 4.4.3. Steps 2–4 were repeated 35X. Annealing temperatures X and primers are presented in **Table 2**.

Table 2. Genes and their respective primer sequences used for PCR and RT-PCR. All primers for both pluripotency and embryoid body germ layer detection are listed in the table. Primers, annealing temperatures (Ann. °C) and amplicon sizes in base pairs (bp) are presented.

Gene	Forward primer	Reverse primer	Size (bp)	Ann. (°C)
GAPDH	agccacatcgctcagacacc	gtactcagcgccagcatcg	302	60
sendai exo-OCT4	cccgaaagagaaagcgaacca	aatgtatcgaaggtgctcaa	483	55
sendai exo-SOX2	atgcaccgctacgcagtgagcgc	aatgtatcgaaggtgctcaa	451	55
sendai exo-KLF4	ttcctgcatgccagaggagccc	aatgtatcgaaggtgctcaa	410	55
sendai exo-C-MYC	taactgactagcaggcttgtcg	tccacatacagtcctggatgatgatg	532	55
exo-EBNA1	ggggtagaggacgtgaaaga	ggtggaaaaatggccttcta	162	60
endo-OCT4	gacaggggggaggggaggagctagg	cttccctccaaccagttgccccaaac	144	60
endo-SOX2	gggaaatgggaggggtgcaaaagagg	ttgcgtgagtgtggatgggattggtg	151	60
endo-REX1	cagatcctaaacagctcgcagaat	gcgtacgcaaattaaagtccaga	306	55
endo-C-MYC	gcgtcctgggaagggagatccggagc	ttgaggggcatcgtcgcgggaggctg	328	60
endo-NANOG	tgcaaatgtcttctgctgagat	gttcaggatgttggagagttc	287	45
SOX17	cgcacggaatttgaacagta	cacacgtcaggatagttgcag	166	55
SOX1	aaagtcaaaacgaggcgaga	aagtgcttggacctgcctta	158	55
AFP	catccaggagagccaagcat	cgccacaggccaatagtttg	209	55
A-cardiac actin	ggagttatggtgggtatgggtc	agtggtgacaaaggagtagcca	486	55
PAX6	aacagacacagccctcacaaaca	cgggaacttgaactggaactgac	275	55
VEGFR2	gtgaccaacatggagtcgtg	tgcttcacagaagaccatgc	218	55

4.4.2.4 Agarose gel electrophoresis

The PCR-products were run on a 1,7% agarose (peqGOLD Universal Agarose; PeqLab) containing 0,01% (V/V) of ethidium bromide (10 mg/ml; Sigma). The products were loaded on gel with 6x DNA Loading Dye (Fermentas) using a 50 bp DNA Ladder (Generuler; Fermentas) as a molecular weight marker. Loaded samples were run for 50 min at 80 V (BioRad and Amersham Biosciences) and gels imaged with UV gel documentation system (UVidoc or Chemi XRS Gel Documentation system; Bio-Rad). The images were processed with Adobe photoshop CC 2017.

4.4.3 Karyotyping

Before collecting DNA samples for karyotyping, each MEF-co-cultured cell line was grown for two passages on feeder-free Geltrex®-surfaces prepared as described in chapter 4.3.2. Samples were collected as described in chapter 4.4.2.2 at p. 12 from lines E1.1M-E3.2M; p. 18 from line S3M; p. 15 from lines S1M and S2M and at p. 14 for E4G. The extracted DNA samples were sent to the Finnish Microarray and Sequencing Centre for karyotyping with KaryoLite BoBs (Product number 4501–0010, Perkin Elmer) (For a more detailed description of the karyotyping method, see Lund et al. 2012).

4.4.4 Embryoid body formation assay

iPS-cell lines E1.2M, E2.1M E3.2M and S2M at p. 17 and 18 were used for the EB formation assay. EB differentiation of the iPS-cell lines was done using floating culture on non-adhesive 12-well plates (MPC treatment; Thermo Fisher Scientific). MEF-layers were removed and remaining iPS-cell colonies scraped into KSR-medium lacking β-FGF and transferred onto the non-adhesive 12-well plates. The cells were cultured at 36,5°C and 5% CO_2 for 6 weeks. Medium for the cells was changed at day 6 followed by medium change every 2-3 days.

The differentiation status of the formed EBs was assessed by RT-PCR of two genes for each germ layer. For the ectoderm SOX1 and Paired box gene 6 (PAX6); for the mesoderm α-cardiac actinin and vascular endothelial growth factor receptor 2 (VEGFR2) and for the endoderm SOX17 and α-fetoprotein (AFP) were studied (**Table 2**).

EBs from three wells were combined, washed with 1X PBS before lysis in RLT buffer containing β-ME. RNA extraction and cDNA synthesis was performed as described in chapter 4.4.3. The master mixes and PCR reactions were similar as for NANOG and SOX2 in chapter 4.4.5, using 50 ng of cDNA with an annealing temperature of 55°C. Steps 2)-4) were repeated

40X. Housekeeping gene GAPDH was used as and endogenic control and H_2O and –RT samples as negative controls.

4.5 Real-time-qPCR

To study the expression of pluripotency genes at an early and later passage, and between different patients, a Taqman-chemistry based real-time quantitative PCR experiment was carried out. cDNA-samples from all MEF-grown, episomally transfected lines E1.1M-E3.2M at passages 3 and 9 (except for line E2.4M at p. 9) were synthesised as described in chapter 4.4.2.1, and studied to detect the expression of pluripotency genes OCT3/4, NANOG, C-MYC and KLF4. GAPDH was used as an endogenic control and –RT samples and H_2O as negative controls.

The PCR master mixes consisted of 7,5 µl of Taqman universal Master mix (2x) (Applied Biosystems), 0,75 µl of gene expression assay (Applied Biosystems) (see **Table 3**) and 1 µl of cDNA with H_2O added to a final volume of 15 µl per reaction. All samples were prepared as triplicates. PCR reaction was carried out with the 7300 Real-time PCR system (Applied Biosystems) and PCR conditions were 1) 50°C, 2 min; 2) 95°C, 10 min; 3) 95°C and 4) 60°C 1 min, repeating steps 3) and 4) 40 cycles. C_t values were determined using 7300 SDS Software (Applied Biosystems) and relative quantification was calculated in MS Excel 2016 using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using equation 1:

$$\Delta\Delta C_t = 2^{-[(C_{t,gene} - C_{t,GAPDH}) - (C_{t,calibrator(avg)} - C_{t,GAPDH(avg)})]} \qquad (1)$$

Statistical analyses were performed with IBM SPSS Statistics 23. The level of statistical significance for all statistical analyses was set to <0.05 (p-value). Because of the relatively small sample size, and the observed skewness and data histogram, a normal distribution of the data could not be assumed for any comparisons, hence only non-parametrical statistical tests were used.

First, the gene expression data was normalised against the housekeeping gene GAPDH and gene expression of all studied lines at p.9 was compared against all lines at p. 3. To test if there are any statistically significant differences in the relative gene expressions between the two passages, a non-parametric Mann-Whitney U test was used.

Table 3. Gene expression assay IDs used for real-time-qPCR

Gene	Gene expression assay ID
GAPDH	Hs02786624_g1
SOX2	Hs00999632_g1
NANOG	Hs02387400_g1
KLF-4	Hs00358836_m1
C-MYC	Hs00153408_m1

Secondly, the differences in gene expression at p.3 and p. 9 for each patient was studied. To do this, data from all lines for each patient was normalised against GAPDH and expression at p.9 was compared to the expression for the same lines at p. 3. The differences in relative gene expressions at different passages was compared using a Mann-Whitney U test. Since there was only two lines in both groups for patient 3, statistical tests were not performed for this patient.

Finally, the expression between patients at the same passage was also studied. Lines were normalised against GAPDH and compared to lines form patient 1 at p.3 and p. 9, respectively. A Kruskal-Wallis analysis was performed to detect statistically significant differences between patients at the same passage.

4.6 Cardiac differentiation

4.6.1 Cardiac differentiation and culture conditions

For cardiac differentiation two lines from the same patient but reprogrammed with different method were chosen for comparison: E2.1M and S3M both at passage 18. Differentiation was performed in co-culture with mouse endoderm-like cells (END2-cells), cultured in END2-medium (DMEM/F-12 (Gibco by Life Technologies) supplemented with 7,5% FBS (Gibco by Life Technologies), 1% NEAA (100x MEM; Gibco by Life Technologies), 2 mM L-glutamine (GlutaMAX™, Life Technologies) and 0,5% Pen-Strep (Sigma Aldrich)) (Mummery et al. 2003).

Before the differentiation, END2-cells were treated with 5µl/ml Mitomycin C (R&D Systems) for 3h at +37°C to prevent mitosis. After the mitomycin C -treatment, cells were counted and re-plated at a density of 175 000 cells/well sterile 12-well plates. Before plating, the wells were

treated with 0,1% gelatin as described in chapter 4.3.1. Cell attachment was verified by light microscopy the next day.

Next, medium for the END2-cells was changed into 0% KO-SR hES medium (KO-DMEM (Gibco by Life Technologies) supplemented with 2.92 mg/ml AA (Takahashi et al., 2003; Sigma Aldrich), 2 mM L-glutamine (GlutaMAX™, Life Technologies), 1% NEAA (100x MEM; Gibco by Life Technologies), 0,5% Pen-Strep (Sigma Aldrich) and 0.1 mM β-ME (Sigma Aldrich)). MEF layer was removed from the iPS-cells, and iPS-colonies transferred onto the END2-cells plated the day before. The number of iPS colonies transferred per well was approximately 40 for line S2M and 52 for line E2.1M. Medium was changed on days 7, 9 and 14. On day 16 the medium was changed into 10% KO-SR hES without AA. After this medium was changed every 2–3 days. Cells were cultured at +37°C and 5% CO_2. Cells were visualized under the light microscope every 1–3 days to observe appearing differentiated, beating areas. When no new beating areas had appeared for over 7 days, the beating cardiomyocyte areas were dissociated for further characterization.

4.6.2 Dissociation protocol for beating areas

On day 30 after beginning the differentiation, three beating areas from each cell line was dissected and processed into single-cell suspension before spinning onto small glass plates. The dissociation protocol was performed by the lab technician. Briefly, beating areas were dissected using a microscalpel and washed with low calcium buffer for 30 minutes at room temperature (RT). Then the cells were dissociated into single cells by treating them with collagenase A (Roche Diagnostics) for 45 minutes at +37°C. After this cells were transferred into KB medium with a high K+-concentration for 1 hour at room temperature. After this the cells were centrifuged and re-suspended in a volume of 150 µl of hES-medium. This single cell suspension was spinned down on a small glass cover slip with the cytospin centrifuge for 5min at a speed of 600 rpm.

4.6.3 Immunocytochemistry

Immunocytochemistry for the cytospinned cells was performed as described in chapter 4.4.1. The primary antibody used was a cardiac specific marker Troponin T (goat cardiac Troponin T IgG; Abcam) at a dilution of 1:2000 and the secondary antibody Alexa Fluor 568 nm (donkey anti-goat IgG (Invitrogen)).

4.6.4 Evaluation of cardiac efficiency

The efficiency of the cardiac differentiation of the two lines was assessed in two ways. Firstly, the number of beating areas prior to dissociation was divided by the total number of differentiated colonies. Secondly, efficiency was assessed from the immunocytochemistry of the cardiac cells. from four randomly acquired immunofluorescence images from both lines by dividing the number of cardiac-specific antibody Troponin T- positive cells with the total cell count (stained by DAPI). Images were acquired using Olympus DP30BW and edited with DP manager software (Olympus).

5. Results

5.1 Characterisation of iPS-cell lines

After establishment and maintenance in culture for several passages, all iPSC-lines were characterised to assess their pluripotency and quality. Characterisation of the iPSCs is performed during the stabilisation phase of reprogramming (David and Polo, 2014). All characterisations in this study were performed between passages 7-17. The expression of exogenic genetic material was studied for episomally derived lines by PCR, and by RT-PCR for the sendai-virally reprogrammed lines. The expression of endogenic pluripotency genes was assessed by RT-PCR. An EB formation assay in floating culture (Itskovitz-Eldor et al., 2000; Takahashi et al, 2007) was performed to assess *in vitro* -differentiation potential. The presence of the germ layers was then confirmed by RT-PCR for two genes of each derm (endoderm, mesoderm, ectoderm). Karyotyping analysis was performed in Turku in the Finnish Microarray and Sequencing Centre. However, not all studied lines were characterised with these methods. Characterisations of all lines included assessment of the absence of exogenic genetic material, and the study of expression of the endogenous pluripotency genes. Karyotyping was also performed for all lines. Other characterisations were performed for only chosen lines described in the materials and methods -section. Also notable, no characterisation of Geltrex®-established line E5G was performed, since the line was lost due to complete differentiation at p. 9. Pluripotency gene expression was also studied with real-time-qPCR.

5.1.1 Morphology

The iPS-cells grew in typical dense, flat colonies surrounded by MEF-cells throughout the experiment. Also a typical high nucleus to cytoplasm -ratio with large nucleoli could be observed.

The iPS-cells formed round-shaped colonies of even color, sometimes with darkened, differentiated material around the edges of the colonies. The degree of differentiated material around the colonies was quite small. However, as reaching higher passage numbers, also a higher degree (although altogether a small degree) of differentiated material was observed. Since all MEF-co-cultured iPSC-lines established a similar kind of morphology throughout the experiment, representative images from only four different lines at various passages are presented in **Figure 6.**

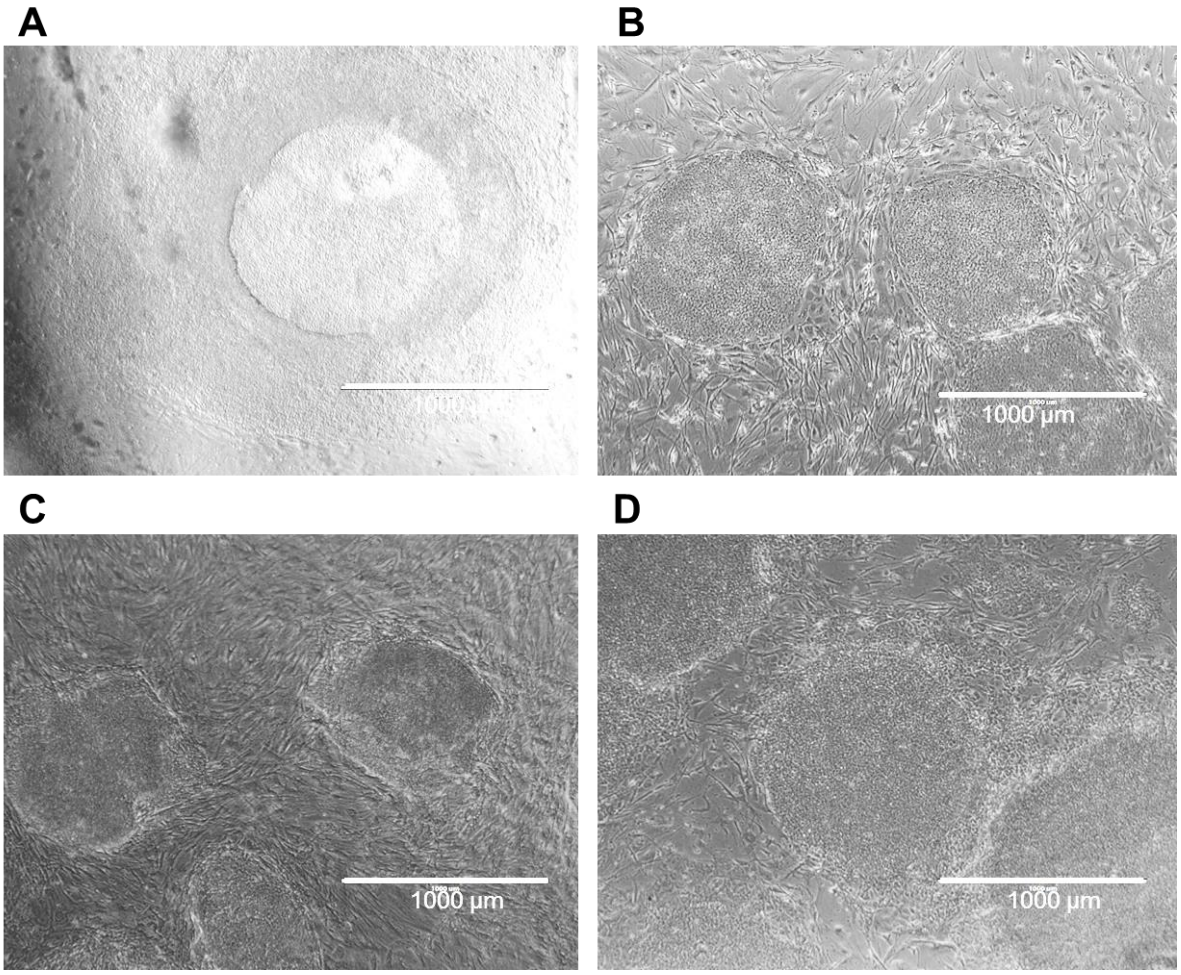

Figure 6. MEF-co-cultured iPS-cell morphologies at different passages. **A)** Line E1.2M at p. 1. A clear, round colony is already visible, but a lot of differentiated material is visible around the edges of the colony. **B)** Line E2.1M at p. 8. Two colonies grown together can be seen on the right. The colonies have a dense and clear structure and are surrounded by MEF-feeder cells. **C)** Line E1.4M at p. 14. Three individual colonies can be seen with a little differentiated material around the edges. **D)** Line E3.2M at p.18. Even at the end of the experiment, the lines were able to elicit a nice morphology with little or no differentiated material around the edges. Colonies are round, flat and even-colored.

5.1.2 PCR and RT-PCR

The absence of exogenic genetic material was confirmed by PCR for electroporated lines and RT-PCR for sendai-lines. For electroporated lines the expression of EBNA-1 gene (present in all the transfection plasmids), and for the sendai-lines of all viral transgenes KLF-4, SOX-2, C-MYC and OCT-3/4 was studied. The positive EBNA-1 plasmid dilution series could amplify the studied sequence from down to 1 pg of plasmid, but not from any of the studied iPS-lines (see **Figure 7A)**. The sendai positive control (RNA from a sendai-line at p. 3) was also able to amplify all the viral transgene sequences, but no viral sequences were detected in the iPS-lines **(Figure 7B)**.

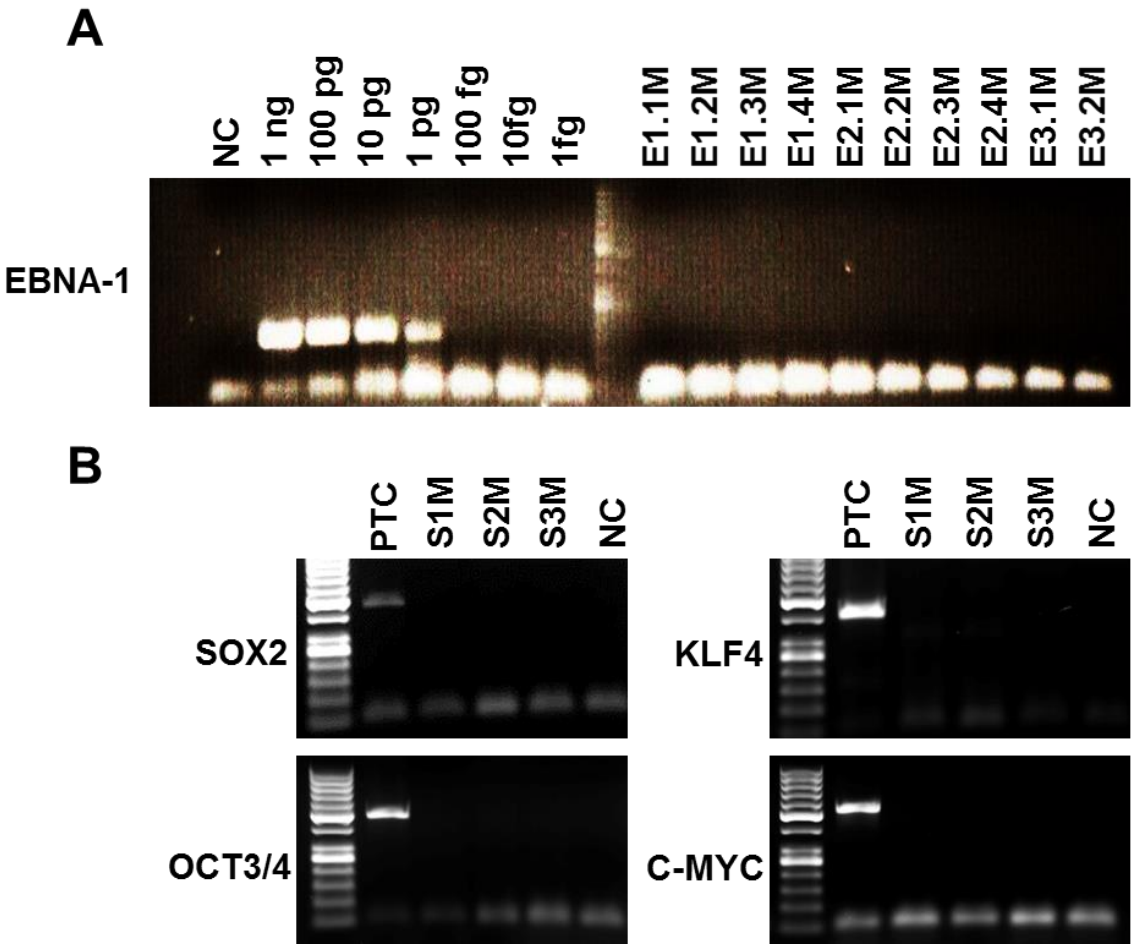

Figure 7. PCR and RT-PCR to detect exogenic genetic material in iPSC-lines. **A)** PCR for the EBNA-1 gene included in the transfection plasmid in electroporated lines showed that no exogenic material was present in iPS-lines at p. 8. NC=negative control (H_2O instead of RNA in the reaction), and as the positive control an EBNA-1 plasmid dilution series. **B)** RT-PCR for viral transgene sequences for KLF-4, SOX-2, C-MYC and OCT-3/4 revealed no viral exogenic material at p. 14 and 15. PTC=positive template control, a sample collected from p. 3 sendai-transfected line; NC= negative control (a negative -RT control with no reverse transcriptase in the cDNA turn). 50 bp DNA ladder.

After verification of the absence of exogenic genetic material, the expression of endogenic pluripotency genes REX1, OCT-3/4, C-MYC, NANOG and SOX-2 was studied at mRNA level by RT-PCR. GAPDH was used as an endogenic control, and negative –RT control (with no reverse transcriptase in the cDNA-turn) or H_2O control (with H_2O instead of RNA in the reaction) for all samples were also studied. All studied iPSC-lines showed endogenic expression of all studied pluripotency genes, except C-MYC for line E2.4M (**Figure 8**). However, a rerun with double the amount of cDNA was able to amplify the gene also for line E2.4M (results not shown). No apparent differences in the expression of pluripotency genes in the sendai-reprogrammed lines compared to electroporation-lines were visible.

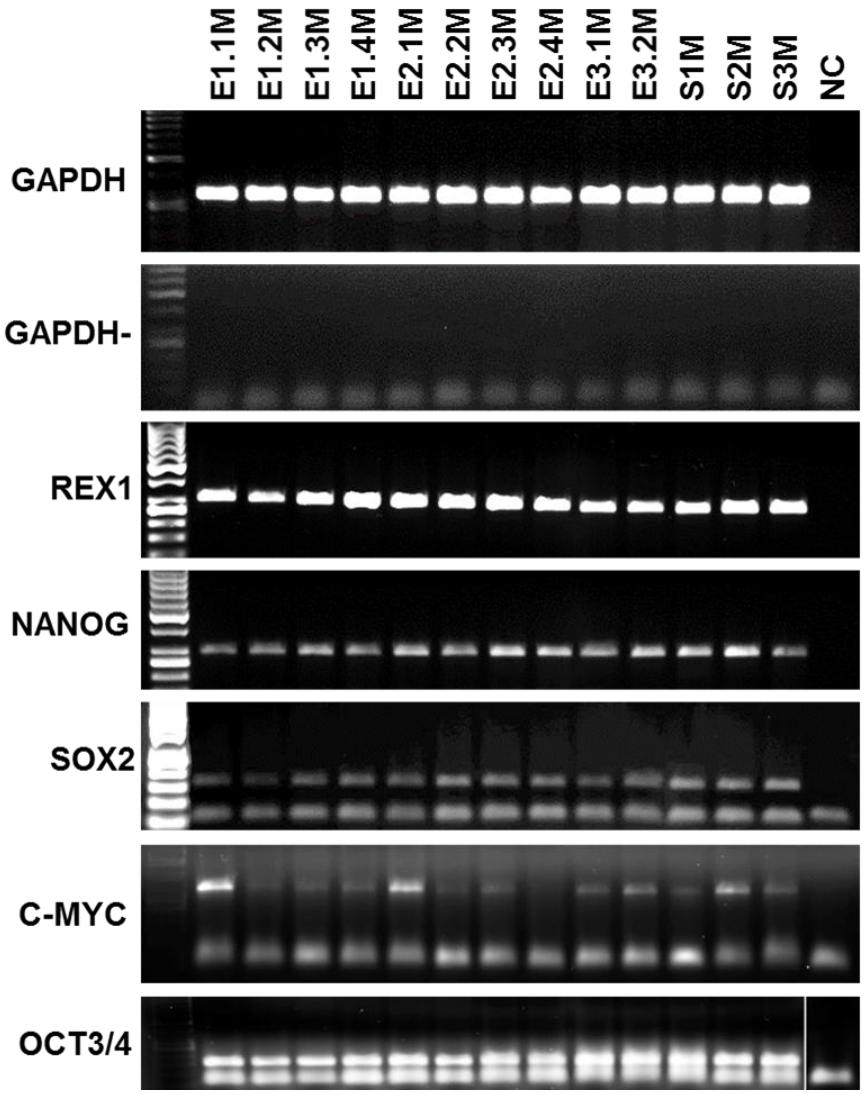

Figure 8. RT-PCR for studying the expression of endogenic pluripotency genes. GAPDH was used as an endogenic control, and a –RT control PCR (with no reverse transcriptase in the cDNA turn) with GAPDH was also conducted. All studied genes REX-1, NANOG, SOX-2, C-MYC and OCT-3/4 were expressed in all lines except C-MYC in E2.4M. Expression of SOX-2 and C-MYC was generally lower than the expression of other genes. NC=negative control (a negative -RT control with no reverse transcriptase in the cDNA turn). 50 bp DNA ladder.

5.1.3 Immunocytochemistry

The expression of pluripotency factors Sox-2, Oct-4, Ssea-4, Tra 1-60 and Tra 1-81 was studied at protein level with immunocytochemistry using DAPI as a counterstain. As seen in **Figure 9**, line E2.1M stained positive for all studied pluripotency factors. Other studied iPSC-lines (E1.2M, E3.2M and S1M-S3M) also expressed pluripotency proteins in a similar manner. No apparent difference in protein expression between the lines was visible, thus results for only one representative line are shown. The results for other lines can be found in **Appendix B**.

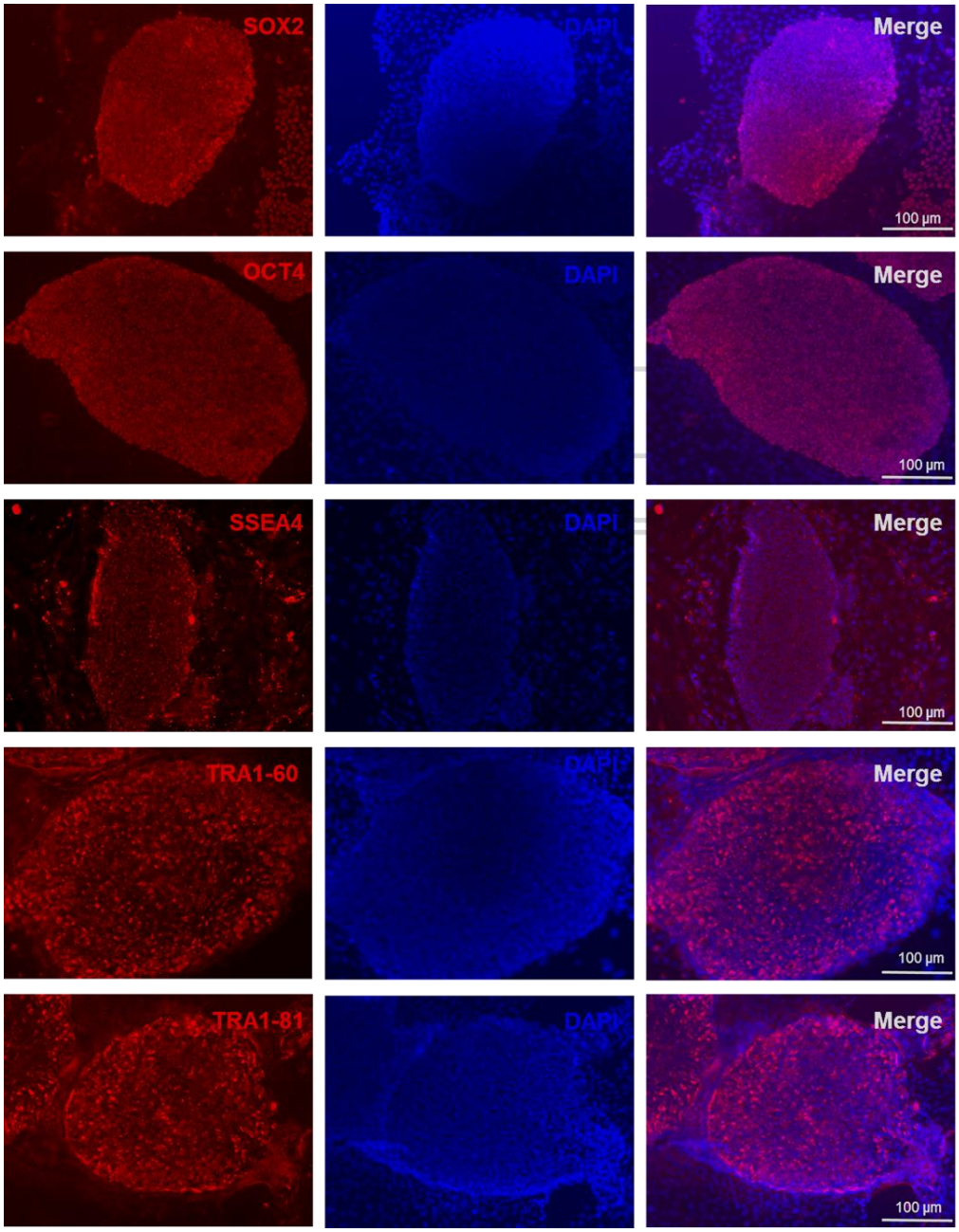

Figure 9. Protein expression of pluripotency markers in iPSC-line E2.1M at p. 10 studied with immunocytochemistry. All used markers Sox-2, Oct-4, SSEA-4, TRA 1-60 and TRA 1-81 stained positive (red). DAPI counterstain was used to stain nuclei (blue) from both iPS- and MEF-cells (stained only blue in the Merge photos).

5.1.4 Karyotyping

DNA samples from all MEF-co-cultured iPS-lines were sent to the Finnish Microarray and Sequencing Centre in Turku for karyotyping. All lines possessed a normal karyotype with no large chromosomal aberrations (data shown only for E4G in chapter 5.2.2).

5.1.5 Embryoid body formation

To assess the in *vitro* –differentiation potential of the iPS-cells, an EB-formation assay was performed for lines E1.2M; E2.1M; E3.2M and S2M. The EBs grown for 6 weeks in floating culture formed cell aggregates of varying sizes with typical pigmented areas (**Figure 10A)**. The differentiation into all three germ layers (endoderm, mesoderm and ectoderm) was studied by the expression of two genes for each derm **(Figure 10B)**. Only for line S2M the expression of all studied genes was detected. Electroporation lines showed no gene expression for mesodermal gene α-cardiac actinin or ectodermal SOX1. Also a second RT-PCR using double the amount of cDNA failed to amplify these genes. Other genes were amplified, and at least one gene for each derm was expressed for all lines. The endogenous control gene GAPDH was amplified in all lines (data not shown).

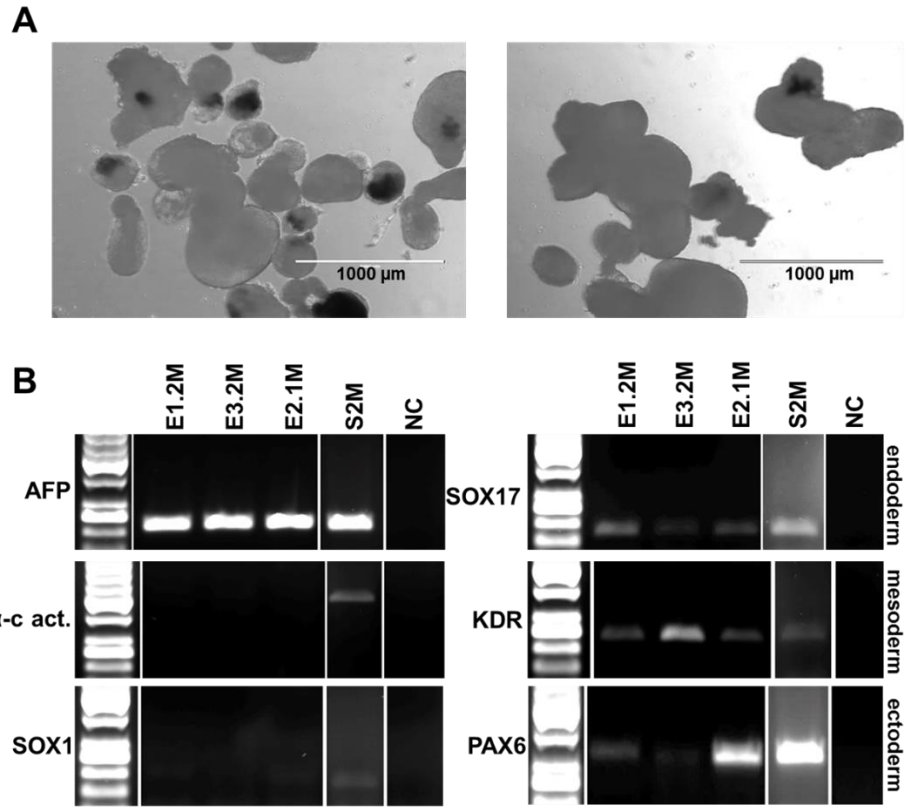

Figure 10. Formation of embryoid bodies and detection of the three germ layers by RT-PCR. **A)** Light microscopy images of embryoid bodies grown for 6 weeks in floating cultures. Line E2.1M on the left and E1.2M on the right, both showed typical pigmented areas in some of the various sized EBs. **B)** RT-PCR for lines E1.2M; E2.1M; E3.2M and S2M for endodermal genes AFP and SOX17, mesodermal genes α-cardiac actinin and VEGFR2 and ectodermal genes SOX1 and PAX6. Not all genes were expressed in all lines except for line S2M, but at least one germ for each germ layer was detected for all. NC=negative control (a negative -RT control with no reverse transcriptase in the cDNA turn). 50 bp DNA ladder.

5.1.6 Quantitative pluripotency gene expression

The relative gene expression was studied by real-time qPCR for pluripotency genes SOX2, C-MYC, NANOG and KLF4. The raw Ct values obtained from the measurements are presented in **Appendix C**.

The relative expression of all genes at p.9 compared to p. 3 for all studied cell lines combined is presented in **Figure 11**. The expression of SOX2, C-MYC and KLF4 was fairly similar at both passages, and no statistically significant differences were observed. NANOG expression at p.9 was statistically significantly lower compared to p.3 (p-value = 0.004, **Figure 11**) when looking at all lines combined.

Since multiple lines form each patient were studied, the pluripotency gene expression for each patient at passages 9 and 3 was compared. Due to small sample size, data for patient 3 was not analysed. The results for patients 1 and 2 are presented in Figure 12. NANOG expression for patient 1 was statistically significantly decreased at p. 9 compared to p.3 (p-value = 0.043; **Figure 12A**), as well as SOX2 expression for patient 2 (p-value = 0.034; **Figure 12B**). Statistically significant differences for other studied pluripotency genes were not observed for either patient.

No statistically significant differences were observed in pluripotency gene expression between patients at the same passage.

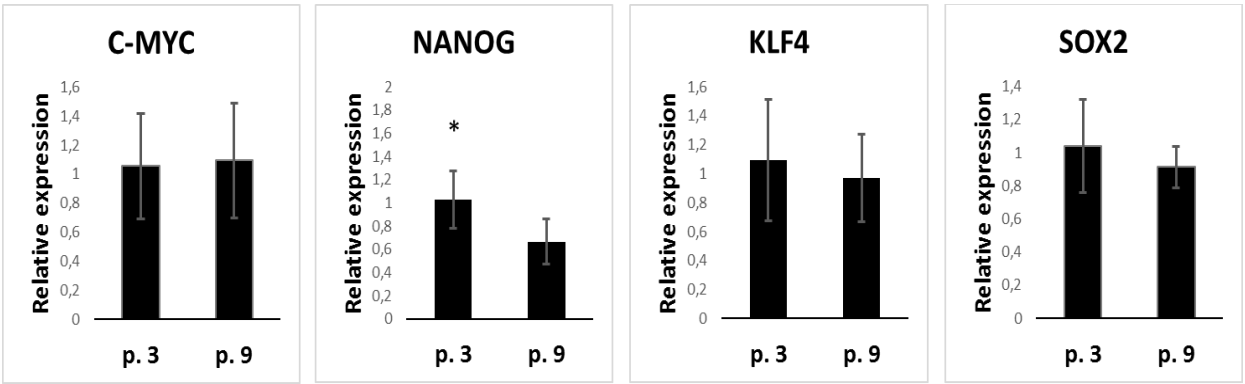

Figure 11. The relative gene expressions of pluripotency genes KLF4, C-MYC, SOX2 and NANOG at passages 3 (p. 3) and 9 (p. 9), assessed by real-time-qPCR. Overall fold changes are small for all genes. No apparent differences in the relative gene expressions can be observed, except for NANOG. Analysed by a non-parametric Mann-Whitney U test the relative expression of NANOG is significantly higher at p. 3 than at the later p. 9. Data is normalised to GAPDH and compared to all lines at p. 3; mean ± SD (standard deviation); N=10 at p.3 and 9 at p.9; * $p < 0.05$.

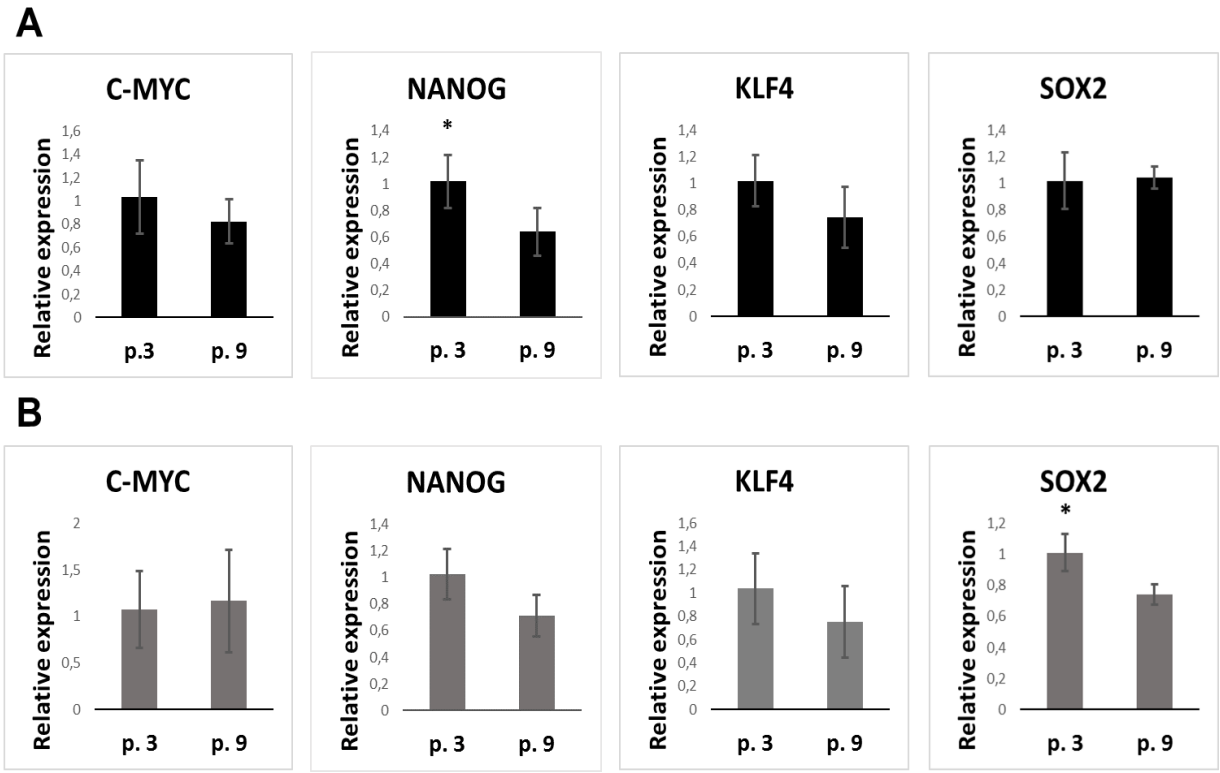

Figure 12. The relative gene expressions of pluripotency genes KLF4, C-MYC, SOX2 and NANOG for patients 1 and 2 assessed by real-time-qPCR. **A)** Relative gene expressions of each gene at passage 3 (p.3) and passage 9 (p. 9) for patient 1. Differences in gene expression between the two passages was studied with a Mann- Whitney U test, and revealed a statistically significant decrease in NANOG expression at p. 9. Data is normalised to GAPDH and compared to all p. 3 lines from patient 1; mean ± SD (standard deviation); N=4; * $p < 0.05$. **B)** Relative gene expressions of each gene at p.3 and p. 9 for patient 2. A Mann-Whitney U test revealed a statistically significant decrease in SOX2 expression at p.9. Data is normalised to GAPDH and compared to all p. 3 lines from patient 2; mean ± SD; N=4; * $p < 0.05$.

5.2 Feeder-free experiment

5.2.1 Morphology and growth

The morphologies of the Geltrex®-cultured lines were somewhat different from traditional MEF-co-cultured iPSC-lines. The morphologies of the individual colonies often tended not to be completely round but had partly sharp edges (**see Figure 13A**), as frequently encountered with feeder-free monolayer cultures (Brouwer et al., 2016). However, also nice, round colonies could be seen (**Figure 13B)**. Also some kind of differentiated cell mass between iPSC-colonies was visible at almost every passage in both lines (**Figure 13C)**. Passaging this cell mass further resulted in early neural network-resembling structures **(Figure 13D).** To preserve nice, even colonies of iPS-cells, Geltrex®-cultured lines required scraping off differentiated cell mass before passaging. Line E4G was also passaged at one point by picking only a part of a good-

looking colony with a pipette tip, thus passaging only that further. Even so, at every passage differentiated cell mass (similar to that in **Figure 13C**) reappeared. Without removal, the amount of differentiated cell mass seemed to increase, and eventually line E4G ended up in a morphology similar to that represented in **Figure 13D** and was lost due to complete differentiation at p. 9. Compared to the MEF-cultured lines, the feeder-free lines had a more rapid growth rate and had to be passaged twice as often as the MEF-cultured lines.

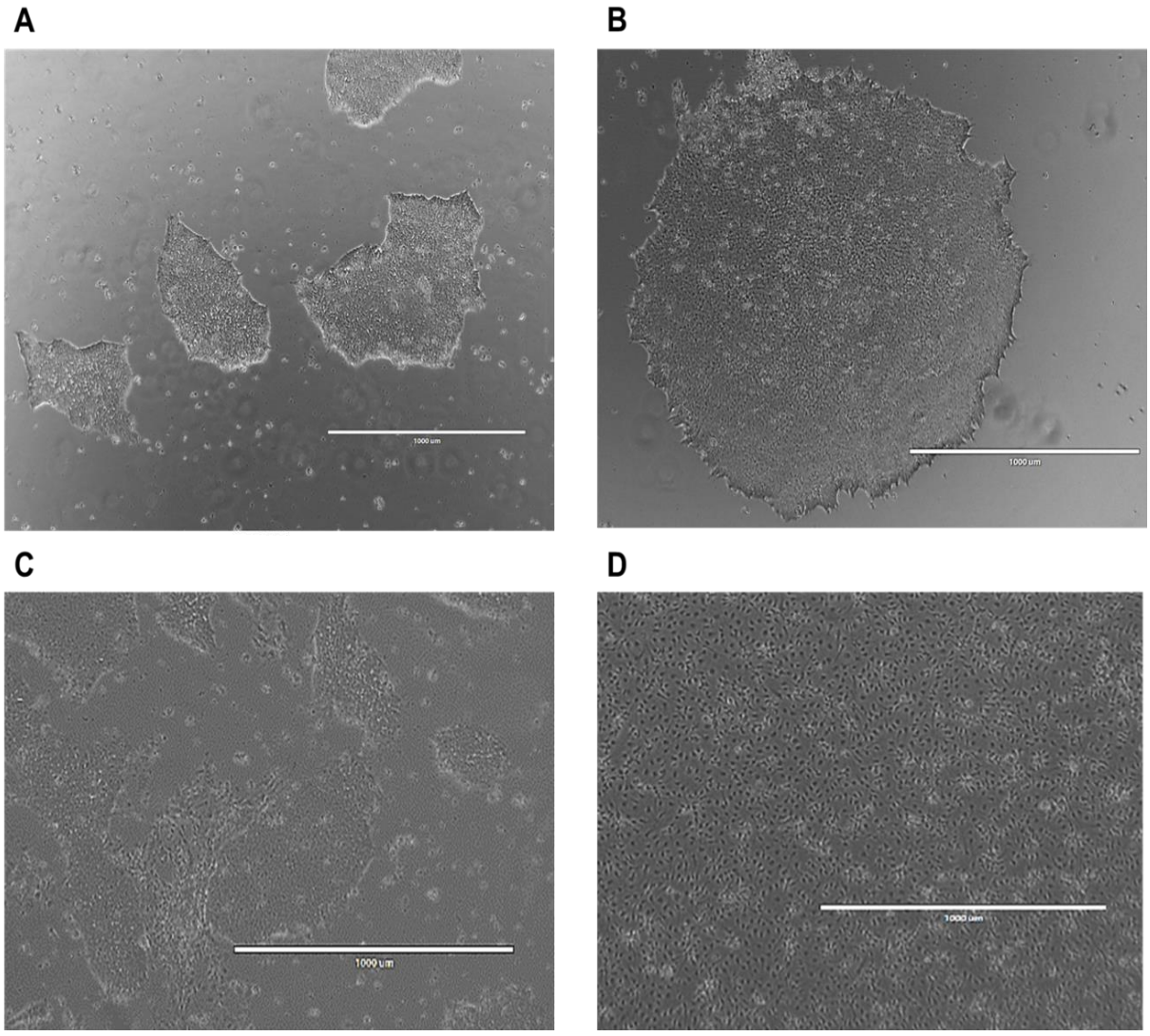

Figure 13. Typical morphologies of the Geltrex®-cultured lines. **A)** Line E4G at p. 14. The iPS-cells formed dense colonies with even color, but with more sharp edges than in lines grown in co-culture with MEF-cells. **B)** Line E5G at p. 1. The Geltrex®-cultured lines were able to form dense, even, round-shaped colonies after picking. **C)** Line E4G at p.6. In between iPS-colonies a differentiated cell mass is visible. Differentiated cells in various amounts appeared at almost all passages in both Geltrex®-cultured lines. **D)** Line E4G at p. 7. Differentiated cell mass passaged further has formed a single, neural-cell-resembling web. No iPSC-colonies are present. Also line E4G was lost at p.9 due to differentiation into cells of similar morphology. Scale bar 1000 µm.

The cells seen in **Figure 13D** and later at p. 17 were stained immunocytochemically with early neural markers Nestin and MAP-2. Both markers stained positive (**Figure 14, B and C**), indicating neural differentiation of the iPS-cells. The differentiated cells that had been passaged on further to develop the morphology seen at. p 7 (**Figure 14A**) formed thin, tubular-like filamentous structures when immunocytochemically stained with early neural marker Nestin. All cells in the well stained positive. Interestingly, another staining with Nestin at p. 17 for the less matured differentiated cell mass among the iPSC-colonies, showed no tubular structures or denser cell areas. Moreover, also iPSC-colonies not expressing Nestin were observed. Colonies not expressing MAP-2 were also seen, indicating that only a part of the iPSCs have differentiated into early neural progenitors.

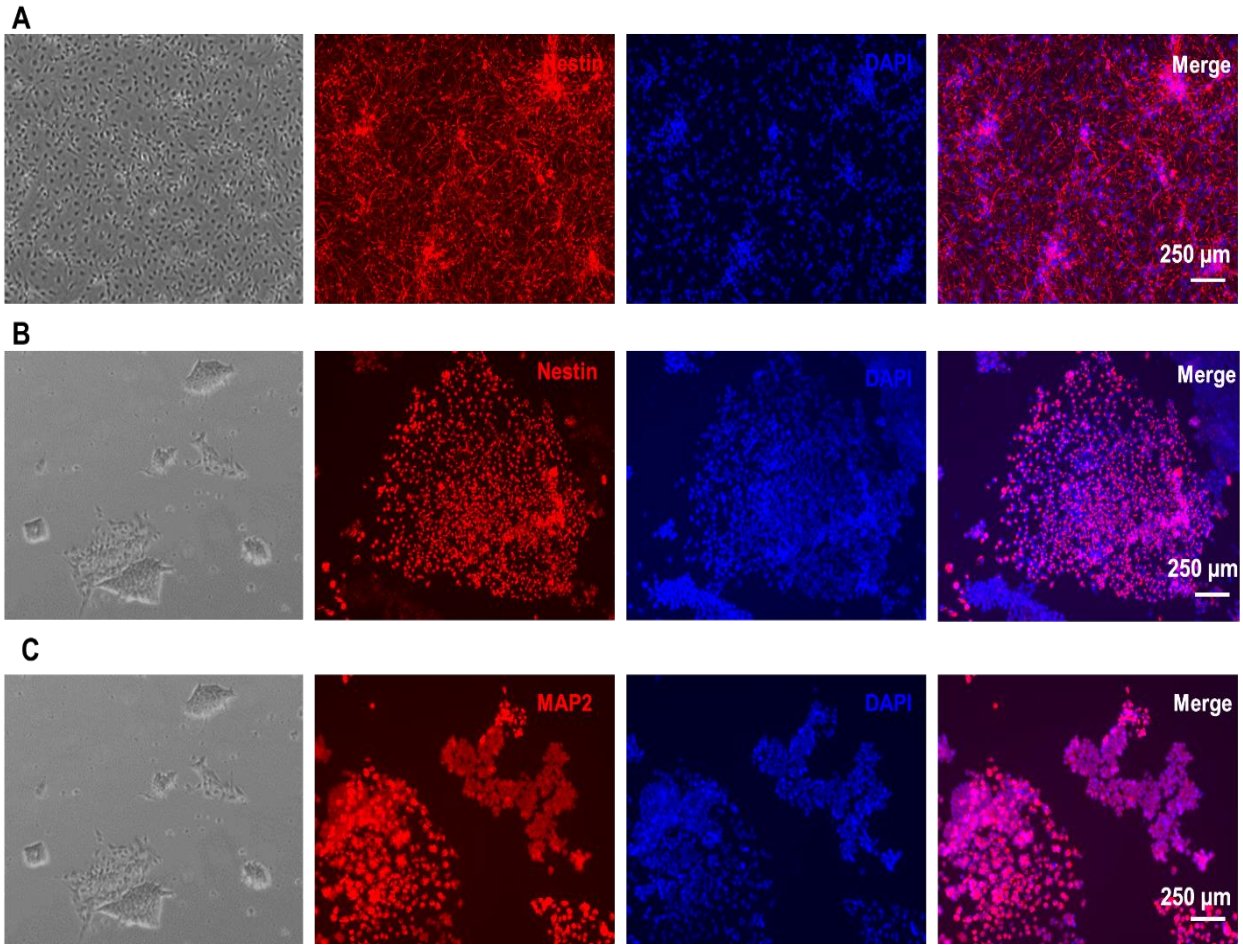

Figure 14. Immunocytochemistry for Geltrex®-line E4G using early neural markers MAP-2 and Nestin (red) and DAPI as a counterstain (blue). Also light microscopy images of the stained cells are presented. **A)** Nestin stained positive at p. 7. Thin, filamentous structures are visible, with denser cell areas. All cells stained positive for Nestin. **B)** Cells stained positive for Nestin also at p. 17 but no filamentous structures nor denser cell areas are visible. iPS-colonies with no neural differentiation were also visible (only stained blue in the Merge-photo) **C)** The iPSCs stained positive also for MAP-2 at p. 17.

5.2.2 Characterisation

Immunocytochemistry for line E4G revealed expression of the pluripotency proteins (**Figure 15**), Line E4G was also characterised by PCR of the EBNA-1 gene to verify the absence of exogenous transfection plasmid DNA material, RT-PCR of endogenous pluripotency genes, by immunocytochemistry of pluripotency markers and by karyotyping. All endogenous genes were expressed at mRNA-level in the cell line (**Figure 16A**), and no exogenous genetic material was detected (**Figure 16B)**. Karyotype analysis performed at the Finnish Microarray and Sequencing Centre revealed a normal karyotype. The karyotyping results can be found in **Appendix D**.

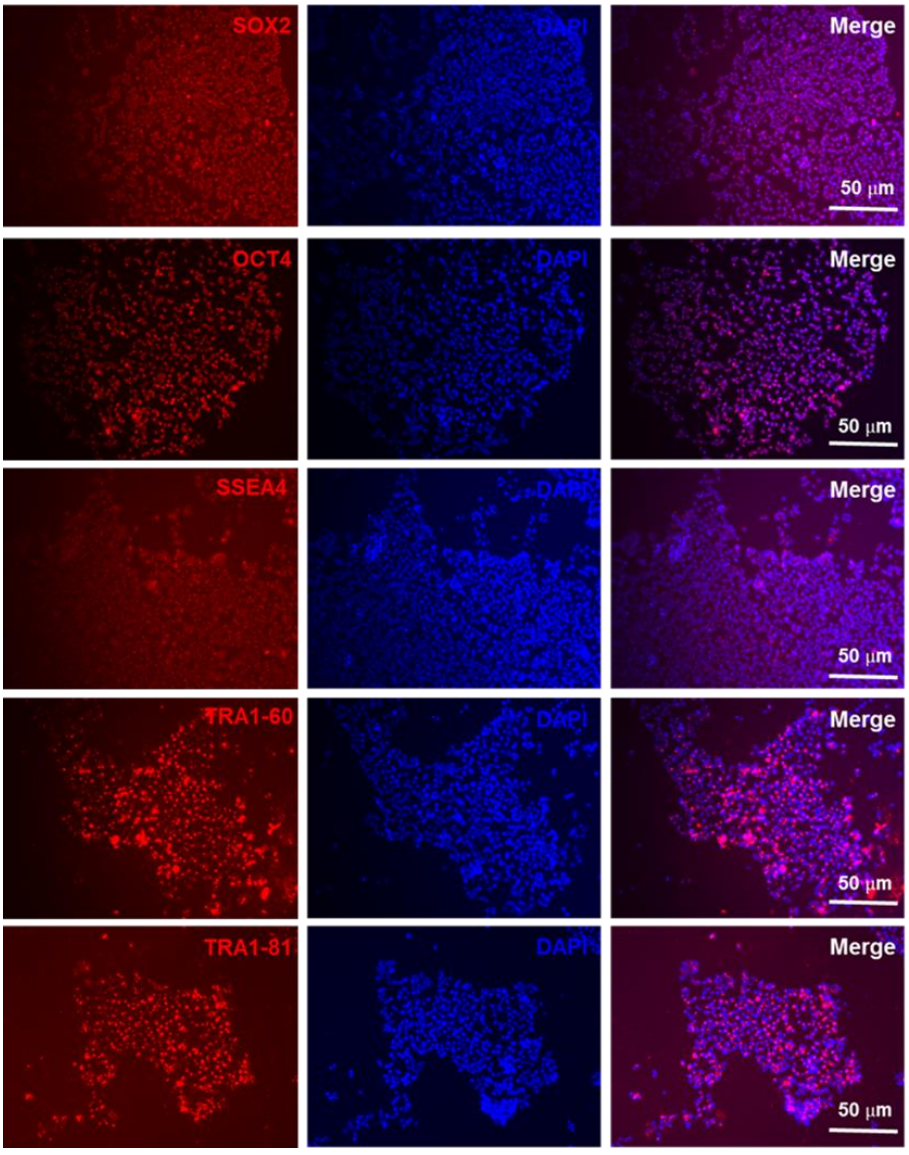

Figure 15. Immunocytochemistry for pluripotency markers Sox-2, Oct-4, Ssea-4, Tra 1-60 and 1-81 for line E4G. The cell line stained positive for all studied proteins (red). DAPI was used as a counterstain. Merge-photos are shown on the right with a scale bar of 50 µm.

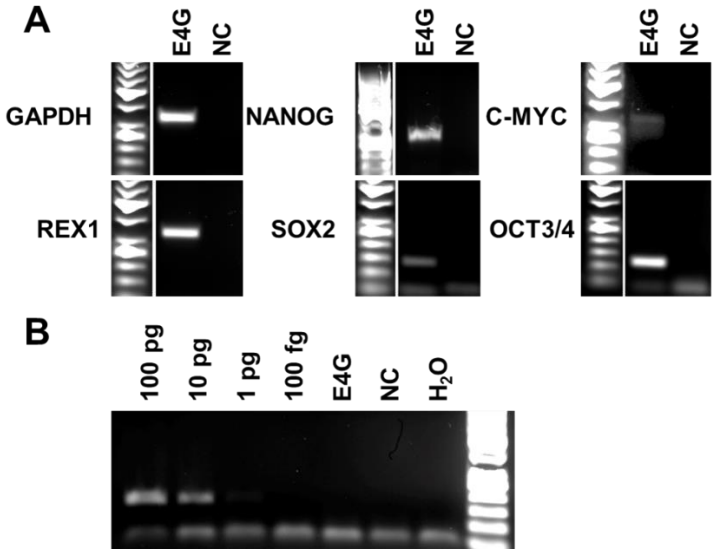

Figure 16. Characterisation of Geltrex®-line E4G. **A)** RT-PCR for endogenous pluripotency genes REX-1, NANOG, SOX-2, C-MYC and OCT-3/4 and an endogenic GAPDH control. All endogenous genes were expressed. NC= negative control, -RT control (a negative- RT control with no reverse transcriptase in the cDNA turn). **B)** PCR for the EBNA-1 gene using an EBNA-1 plasmid dilution series as a positive control. Nothing amplified from line E4G even though DNA from 10pg of plasmid gave a clear band. NC=negative control (a negative control with no DNA polymerase in the reaction). 50 bp DNA ladder.

5.3 Reprogramming efficiency

iPS-reprogramming efficiency was studied for episomal lines from patients 1-5. The efficiency was assessed in two ways: after transfection, all appearing colonies were counted and divided by the number of cells used for transfection, as well as by dividing the number of colonies that survived after picking with the number of colonies picked. Efficiencies are presented as percentages. Results are shown in **Table 4**.

Table 4. Reprogramming efficiency for electroporation lines generated from patients 1-5**.**

	Transfected Cells	Colonies	Efficiency %	Picked Colonies	Successful Colonies	Efficiency %
Patient 1	600 000	96	0.016	35	20	57.1
Patient 2	600 000	31	0.005	13	9	69.2
Patient 3	600 000	100	0.017	24	13	54.1
Patient 4	500 000	8	0.002	7	4	57.1
Patient 5	500 000	21	0.004	14	11	78.5

Transfection efficiencies remained very low, in the range of 0,005–0,017% for all lines, assessed by the number of colonies that appeared after transduction of the reprogramming factors. The survival rates of picked colonies was much higher, but no notable differences between the Geltrex®-established or MEF-established lines could be seen. Variability between patients was as big as variability between the two establishment methods. No statistical analysis was performed because of the small sample size.

5.4 Cardiac differentiation and efficiency

Cardiac differentiation was performed for lines E2.1M and S2M. The cardiac differentiation was assessed by visually inspecting beating areas in co-cultures with END2-cells and by immunocytochemistry of three dissected and dissociated beating areas with cardiac-specific protein Troponin T (**Figure 17**). The first beating areas appeared on days 9 (S2M) and 8 (E2.1M) of the differentiation protocol. Beating areas may still appear even at day 30 of the differentiation protocol but since no new beating areas had appeared for over 7 days at day 30, the beating areas were dissected, spinned down on glass cover slips and stained with a cardiac-specific marker Troponin T.

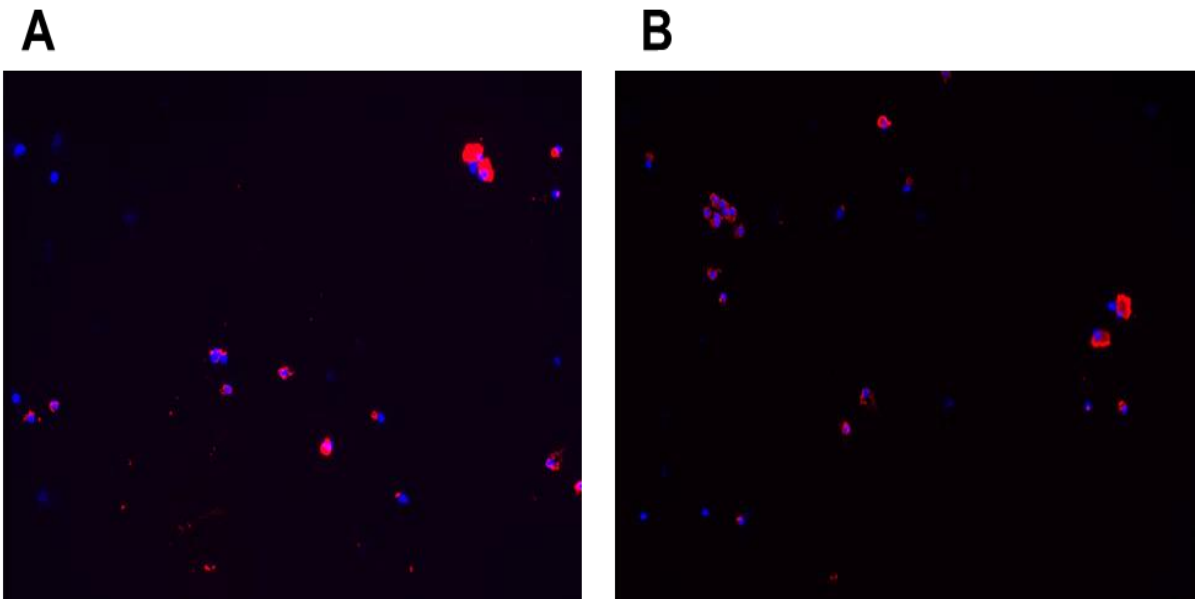

Figure 17. Cardiac differentiation and efficiency assessed by immunocytochemistry with cardiac-specific Troponin T. Four images acquired randomly from both lines and the ratio of cardiac Troponin T –positive cells (red) to total cell count (nuclei stained blue by DAPI) was used to assess the differentiation efficiency. Efficiency was also calculated as the ratio of beating areas appearing in the END-2 co-cultures to total number of colonies transfected. One image for both cell lines is shown. A) Cardiac Troponin T- staining for line E2.1M. B) Cardiac Troponin T- staining for line S2M.

The differentiation efficiency between the two lines was assessed in two ways. Firstly, by comparing the number of beating areas divided by the number of iPSC-colonies used for differentiation (see Table 5), and secondly by immunocytochemical methods. Four images from both Troponin T-labeled lines were taken randomly, and the Troponin T-positive cell count was divided by total cell count (given by counterstaining with DAPI). The results are shown in Table 5. Overall, differentiation efficiency by the number of beating areas remained quite low, 2.5% and 4.6%. The efficiency of the sendai-line S2M, however, was almost twice as high as for the episomal line E2.1M. Also by the amount of Troponin T -positive cells the sendai-line was superior, although the difference was not as big as with the beating area count. In conclusion, assessed by both methods, the sendai-line S2M differentiated more efficiently compared to the electroporation line E2.1M from the same patient.

Table 5. Cardiac differentiation and efficiency. The beating areas formed during the differentiation and the number of iPSC-colonies originally used for differentiation. Efficiency$_1$ is calculated as their ratio. From the immunocytochemistry, the Troponin T –positive cells and the total amount of cells were counted, and the efficiency calculated as their ratio. Efficiencies are presented as percentages.

	Colonies	Beating areas	Efficiency %	Total cell count	Troponin T - positive cells	Efficiency %
E2.1M	588	15	2.5	55	29	52.7
S2M	241	11	4.6	89	60	67.4

6. Discussion

6.1 iPSC-generation efficiency

The differentiation efficiencies in this study were compared between MEF- and, and Geltrex®-established lines. Although in the original paper by Okita et al. reprogramming by episomal plasmids was enhanced by 10-100-fold by the addition of p53 suppressor (Okita et al., 2011), with efficiencies of approximately 0,03% or higher, the use of the same reprogramming method in this study resulted in modest reprogramming efficiencies in the range of 0,005–0,017% (**Table 4**). However, it was possible to generate iPSC-lines by this method from all patients even in feeder-free conditions. Although viral methods are usually considered more efficient (Brouwer et al., 2016), the lower reprogramming efficiencies are not as important as the generation of higher-quality, integration-free iPSCs with no risk of insertional mutagenesis or transgene reactivation.

The efficiency of reprogramming was assessed both by the number of colonies appearing after transduction as well as the number of colonies surviving after the first picking. Neither of these methods revealed any trends in reprogramming efficiency between the Geltrex®- and MEF-cultures lines. The highest and lowest efficiencies by colony number were obtained for MEF-lines, and no meaningful differences in the efficiencies on colony survival between the two culturing methods were observed, suggesting that variability in the genetic or epigenetic profiles between patients affects the reprogramming method more than the culture conditions used in this study.

6.2 Pluripotency of iPSC-lines

The generation of integration-free iPSC-lines with no detectable trace of the ectopic expression is important for the generation of high-quality iPSC-lines. Integrating methods can result in insertional mutagenesis hampering the normal function of the cells (Brouwer et al., 2016). Moreover, if reprogramming factors persist in the iPS-cells even after differentiation, the reactivation of these transgenes can result in de-differentiation and tumorigenic formation (Okita et al., 2007), which is problematic especially when considering the clinical use of iPSCs. Although considered as a non-integrating method, iPSC-lines generated by plasmid transduction have been reported to integrate into the host cell genome (Montserrat et al., 2011). Moreover, Yu et al. generated lines using a single transduction of three oriP/EBNA-1 plasmids

containing OCT4, SOX2, NANOGg, KLF4; OCT4, SOX2, SV40 large T antigen; and C-MYC and LIN28 and reported that only 1/3 of the subclones lost the episomal plasmids (Yu et al., 2007). However, more recent studies also show successful loss of plasmid during extended periods of culturing. Hu et al. reported plasmid-free lines at p. 15 (Hu et al., 2011), as Chou et al. reported similar results at p. 15 (Chou et al., 2011). In line with these results, no plasmid DNA could be detected by PCR of the EBNA-1 gene in this study. All generated, MEF-cultured iPSC-lines were transgene-free already at p. 8. Furthermore, also the Geltrex®-cultured line E4G failed to amplify DNA of the EBNA-1 gene (**Figure 16B**)**,** indicating the loss of the reprogramming plasmids.

In addition to the episomal plasmid reprogramming method, also the sendai-viral transfection of the reprogramming factors as negative-sense single-stranded RNA is considered an integration-free method. The viral RNA will not enter the nucleus and will usually be diluted out of the cells by p. 10 after the infection (Malik and Rao, 2013). Studied by RT-PCR at p. 14 and 15, the sendai-virally reprogrammed iPSC-lines also failed to amplify sequences for any of the viral transgenes in this study (See **Figure 7B**). Thus, all characterised lines studied were free of ectopic reprogramming factors, independent of the generation method or culturing conditions.

After verification of the absence of ectopic reprogramming factors, the expression of endogenous pluripotency genes REX1, NANOG, SOX2, C-MYC and OCT4 was studied by RT-PCR. All the studied iPSC-lines expressed all studied pluripotency genes (see **Figures 8** and **16A**), indicating successful reprogramming. Furthermore, immunocytochemical staining of pluripotency-related surface antigens SSEA-4, tumor-related antigen (TRA)-1-60 and TRA-1-81 and pluripotency-related proteins Oct3/4 and Sox2 were positive for all studied lines. Thus, in addition to being transcribed, the mRNAs of pluripotency genes are also being translated into protein. The protein expression assessed by the immunocytochemical staining appeared similar in the episomal and sendai lines studied.

Genomic instability in pluripotent stem cells has been detected in ESCs since the early 2000, and is also a major concern in iPSC-lines (Ruiz and Fernandez-Capetillo, 2015). The forced expression of transcription factors required for reprogramming has been linked to an accumulation of various genomic aberrancies such as whole-chromosome aneuploidies, sub-chromosomal deletions or amplifications, point mutations and copy number variants. The

mechanisms causing the genomic instability remain largely unknown (Ruiz and Fernandez-Capetillo, 2015). Since the accumulation of different genomic aberrations may alter cell behavior, they could dramatically distort experimental results and thus affect scientific conclusions (Weissman et al., 2001). To verify that no large chromosomal aberrations are present in the generated iPSC-lines, DNA samples from each line were sent to the Finnish Microarray and Sequencing Centre in Turku for karyotyping. Although all lines expressed a normal karyotype, the absence of smaller genetic aberrations cannot be verified by this method.

iPSCs should be able to spontaneously differentiate into all three germ layers (endoderm, mesoderm and ectoderm) when grown in suspension culture (Itskovitz-Eldor et al., 2000; Takahashi et al., 2007). The presence of all the three dermal layers was studied by RT-PCR using two genes for each derm. Three episomally derived lines and one sendai-line were characterised. While the sendai-virally generated line was expressed all studied genes, the episomal lines expressed only some of the studied genes (See **Figure 10**). However, expression of at least one gene for each derm was detected, indicating that also these lines could differentiate into all three germ layers. In addition to the characterisation of this *in vitro* -differentiation potential, further characterisation of the *in vivo* -differentiation potential could be performed by a teratoma assay in immunodeficient mice.

6.3 Maintenance of pluripotency in culture

The most important thing for the PSC culture conditions is to maintain the undifferentiated, pluripotent state of the stem cells. While traditional culture methods relying on serum- or knock-out serum conditions and MEF-cells usually work comparatively well in maintaining the undifferentiated state of the PSCs, they possess two key problems. In addition to the feeder cells of mouse origin, also the media usually include xenogenic components. Moreover, the factors secreted from MEF-cells to maintain the pluripotency, as well as factors in serum-containing medium are not fully defined. Being able to establish GMP-standardised culture conditions for PSCs requires fully defined conditions. In addition, the xeno-free culture conditions are of importance especially if planning therapeutic use of the iPSCs.

Numerous different studies report successful maintenance of pluripotent stem cells in feeder-free culture conditions using Matrigel and mTeSR1 medium (Akopian et al., 2010; Hakala et al., 2009; Ludwig et al., 2006). Geltrex® is very similar to Matrigel, and both are secreted extracellular matrix proteins purified from murine Engelbreth-Holm-Swarm (EHS) tumor cells.

While Geltrex®-cultured line E4G used in this study expressed pluripotency markers similarly as MEF-cultured lines at mRNA and protein level (**Figures 15 and 16A**), indicating that iPSCs could be generated and maintained in culture for extended periods, it is also noteworthy that a higher degree of differentiated cells was present at all passages when compared to MEF-cultured lines. Moreover, the other Geltrex®-cultured line E5G was completely lost due to differentiation at p. 9. At p. 7 and 17, line E4G stained positive for early neural markers MAP-2 and Nestin. Based on the similar morphology of the cells, also line E5G would most probably have stained positive for these markers (**Figure 14**). These results suggest that culturing in feeder-free conditions using Geltrex® and mTeSR1 medium leads to a high degree of differentiated cells of the neural lineage. Upon culturing these cells further for a few passages, they mature forming a tubular network not visible at less mature cells still expressing Nestin and MAP-2. Since Nestin is an intermediate filament present in early neural progenitors but ultimately lost upon complete differentiation into a certain neural cell type, the presence of Nestin in the more matured cells can indicate two things. Either the progenitors could have terminally differentiated into neural subtypes in prolonged culture, or the culturing conditions used support the differentiation into early neural progenitor but suppresses further commitment of these cells.

The neural differentiation observed in this study is in line with the findings of Ojala et al. (Ojala et al., 2012). Upon long-term culture of five hIPSC-lines, they also encountered more differentiation in the feeder-free cultures on Matrigel in mTeSR1 medium than in the traditional MEF-cultures. Differentiation into the neural lineage was confirmed with cytometric analysis of polysialylated-neural cell adhesion molecule (PSA-NCAM) and immunocytochemistry for MAP-2. Moreover, one of the five lines cultured on Matrigel was also lost after 7 passages because of differentiation, as was also observed for this study.

To maintain a pluripotent-cell morphology in feeder-free culture, the differentiated cells needed to be removed very carefully before passaging. Even when doing so, differentiated cells appeared to some degree at every passage. If not removed, their amount seemed to increase in time as also indicated by the loss of line E4G. Compared to the traditional culture methods including MEFs and knock-out serum replacement-based medium supplemented with β-FGF, the feeder-free method resulted in more spontaneous differentiation. In maintaining the pluripotency of the iPSCs, the traditional method can be considered better than the feeder-free conditions used in this study.

This study did not give answers to whether the neural differentiation arises from the matrix material or the medium. However, as the essential function of the MEF-cells is to provide the cytokine leukemia inhibitory factor (LIF), which directs PS-cell self-renewal through activation of the latent transcription factors STAT3 (Matsuda et al., 1999), the differentiation observed is more probably caused by components or by the absence of specific components in the medium than in the matrix. However, LIF alone is insufficient to prevent neural differentiation. A study by Ying et al. found that a N2B27 medium supplemented with LIF and BMP, however, could prevent neural differentiation of ES-cells in feeder-free culture on gelatin (Ying et al., 2003). Supplementing the culture media with these or other components and their ability to prevent neural differentiation into the neural lineage might reveal some answers to this question. Also other small molecules known to prevent neural differentiation might result in better maintenance in these culture conditions.

Since both the medium and matrix material used in this study are derived of animal origin, the issue of xenogenicity is not prevented using these feeder-free conditions. Moreover, mTeSR1 is not a fully defined medium. Since more differentiation is encountered in these conditions compared to the traditional method, the only true advantages compared to traditional MEF-culturing observed in this study were the faster growth of iPSCs, less laborious passaging and depending on the use of the iPSCs, the absence of cells form another origin from the cultures. However, for *in vitro* and research purposes not requiring xenogenicity or fully defined conditions, the traditional MEF-culture method seems superior to the feeder-free method used in this study.

6.4 Pluripotency gene expression

The expression of pluripotency genes was studied at an early and later passage. Since the activation of the endogenic pluripotency genes in the iPSCs is thought to be a slow process (David and Polo, 2014), the aim was to study at which passage the pluripotency gene expression is upregulated. However, first RNA samples were obtained from passage 3, up to which point the expression had already reached levels similar to that observed at a later passage (**Figure 11**). Interestingly, a statistically significant decline from passage 3 to passage 9 was observed for NANOG (**Figure 11)**. Nanog is a transcription factor regulated by Oct4 and Sox2 (Rodda et al., 2005). It has a role in maintaining the pluripotency through inhibition of the (BMP)-signaling pathway (Suzuki et al., 2006). The expression of SOX2 was statistically significantly also decreased for patient 2 at the later passage (**Figure 12B**), which are in line with the

observed NANOG decline. However, these results were not observed for the other patients. Thus, the downregulation of NANOG could also be induced by OCT4, which was not studied in this thesis.

Downregulation of NANOG has been shown to lead to differentiation in the ES-cells (Hyslop et al., 2005). However, all iPSC-lines were successfully maintained for almost ten passages even after this observed decline of NANOG expression, suggesting that although statistically significant, the decrease would not be sufficient to cause differentiation. Moreover, although statistically significant differences were observed, the fold changes in gene expression were small for all genes. This would indicate that the differences observed would more likely be caused by variation between individual iPSC-lines, which is frequently observed rather than meaningful differences in gene expression. In addition, also sample sizes especially between the different patients and patients at different passages were small.

Although the activation of pluripotency genes is a time-consuming process, already at passage 3 the pluripotency genes are expressed at levels comparable to a later passage. For obtaining more meaningful results, RNA from earlier passages could be used. Fold changes observed for all genes and lines were relatively small, indicating that even if statistical differences between lines could be observed, they may not be meaningful. Furthermore, as lines were reprogrammed by episomal plasmids that also express the studied pluripotency genes SOX2, C-MYC and KLF-4, and the absence of the plasmids was not confirmed at passage 3, it is impossible to know whether the gene expression is a result of only endogenous expression or a combination of endogenous+ plasmid expression, or only plasmid expression. However, NANOG was not included in the episomal plasmids, the expression of which can be assumed as completely endogenous.

6.5 Cardiac differentiation

Two iPSC-lines generated from the same patient with different methods were differentiated into cardiomyocytes by END-2 co-culture. The overall efficiencies were assessed by the number of beating areas appearing in the cultures and by the number of Troponin T-positive cells in these areas (Toivonen et al., 2013). While efficiencies as high as 25% have been reported (Graichen et al., 2008), the efficiencies observed in this study of 2.5% and 4.6% were fairly small even if a known differentiation enhancer AA and serum-free conditions were used (Passier et al., 2005; Takahashi et al., 2003). Better yields could be obtained by supplementing

the medium with p38 MAPK inhibitor, as originally reported in the study achieving 25% differentiation efficiency (Graichen et al., 2008).

Since both lines chosen for comparison were maintained in same culture conditions and were used at the same passage, the differences between the efficiencies should reflect differences due to the reprogramming method. Assessed by both methods the sendai-viral line differentiated into cardiomyocytes more efficiently than the episomal-line with almost twice as high efficiency assessed by the number of beating areas (**Table 5**). These results would suggest that better differentiation efficiencies could be achieved with the use of sendai-virally reprogrammed iPSCs. However, since only two lines were compared, also variation observed between the different iPSCs-lines could explain the better reprogramming efficiency of line S2M. To achieve more significant results, more lines should be studied. However, since the differentiation efficiencies are usually overall very modest with the END2- method, a better way to increase the efficiency might be to choose another differentiation method, such as the 2D-monolayer culture method, for which efficiencies as high as 95 % have been reported (Burridge et al., 2014). However, the END-2 co-culture method is the most robust, and easy method since differentiation is dependent on signals secreted by the END-2 cells, and not much information on the factors facilitating differentiation are needed. However, to create iPSC-derived cardiomyocytes for possible future clinical use, a defined method with known and xeno-free components should be used.

7. Conclusion

One of the main aims of this study was to establish and characterise new iPSC-cell lines. Lines were generated with two different methods: either with sendai-viral reprogramming or episomal plasmid reprogramming with oriP/EBNA-1 plasmid system. In addition, two different culturing conditions were used: the traditional MEF-culturing and a feeder-free Geltrex®-culture method. While all studied patient cells were successfully reprogrammed into iPSC-lines according to the characterisations used in this study, the feeder-free lines elicited a much greater degree of differentiation in culture. Moreover, one of the two feeder-free iPSC-lines was completely lost due to differentiation. Since the feeder-free conditions used still contain xenogens and undefined components, as such it is ill-suited for regenerative medicine purposes or other applications requiring fully defined and xeno-free conditions. If aiming for research purposes not demanding these, the traditional MEF-culture method is considered superior to the feeder-free method. The few advantages achieved with feeder-free culturing were easier passaging and more rapid growth.

The aim of gene expression studies was to assess at which point the pluripotency genes are activated during reprogramming. However, the first RNA samples were obtained only from p. 3 at which point all the studied genes were already expressed at levels comparable to the later passage. Although a statistically significant difference for NANOG expression was observed (expression being lower at the later passage), the overall fold changes in gene expression were small. Since OCT4 is known to regulate NANOG, studying the expression of OCT4 could possibly have revealed more insight into the decreasing NANOG expression.

Reprogramming efficiency was assessed for all episomally derived lines, cultured either in MEF- or feeder-free-cultures. Results of 5 patients revealed no difference in the differentiation efficiencies between culturing methods. Variability in the epigenetic or genetic states between the patients is more likely to cause the differences in reprogramming efficiency.

In addition to reprogramming efficiency, also the cardiomyocyte differentiation efficiency was assessed by two methods for two lines derived from the same patient reprogrammed either sendai-virally or with the episomal plasmid -method. Results revealed more efficient differentiation of the sendai-virally derived iPSC-line. Thus, sendai-virally reprogrammed lines might differentiate more easily into cardiomyocytes. However, only two lines were chosen for

comparison and results from multiple lines would be needed to fully establish the superiority of the sendai-viral method for cardiac differentiation of iPSCs. Differentiation efficiencies observed in this study were low, both under 5%. Better efficiencies could be obtained by supplementing the medium with known enhancers or using another differentiation method such as a 2D monolayer culture method.

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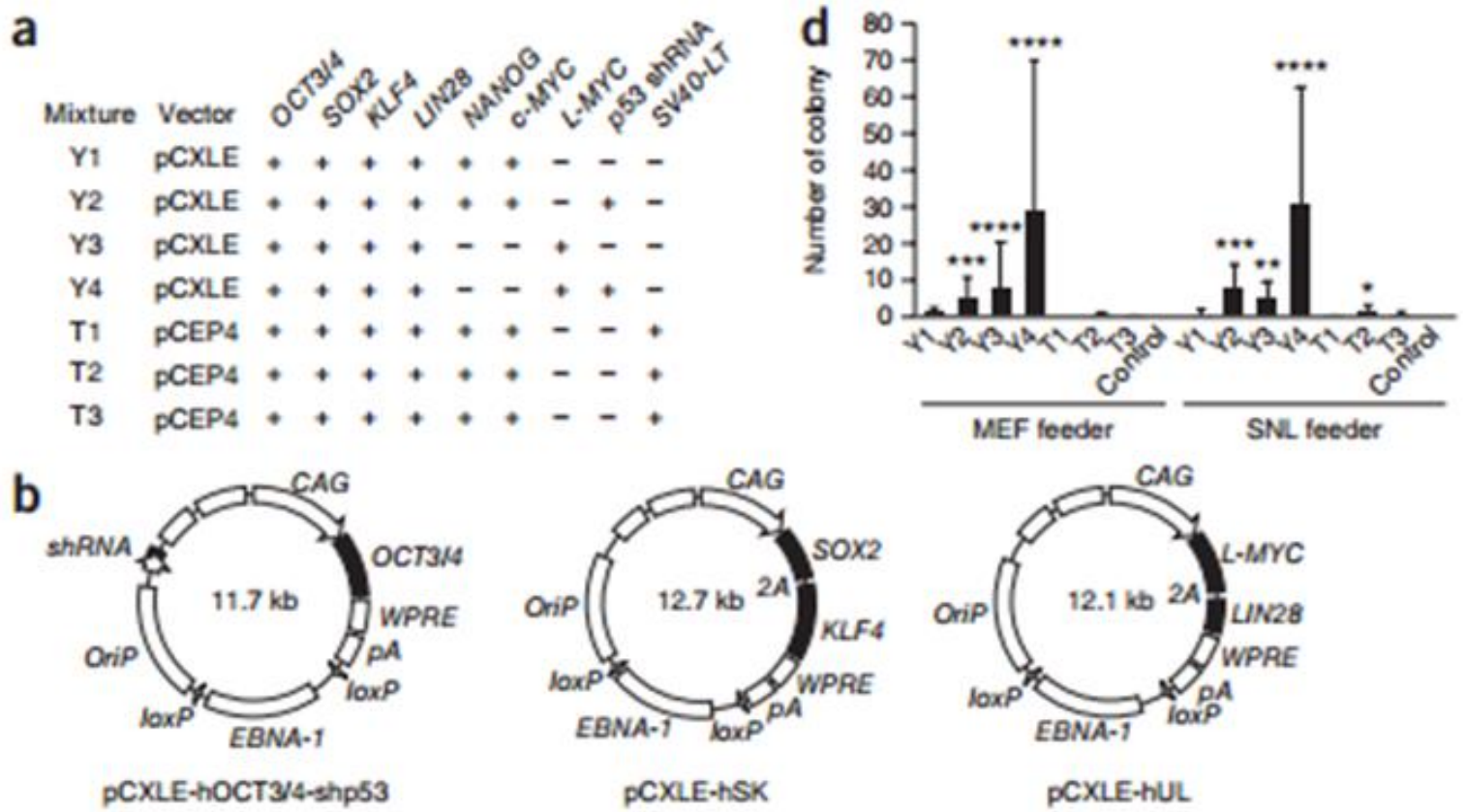
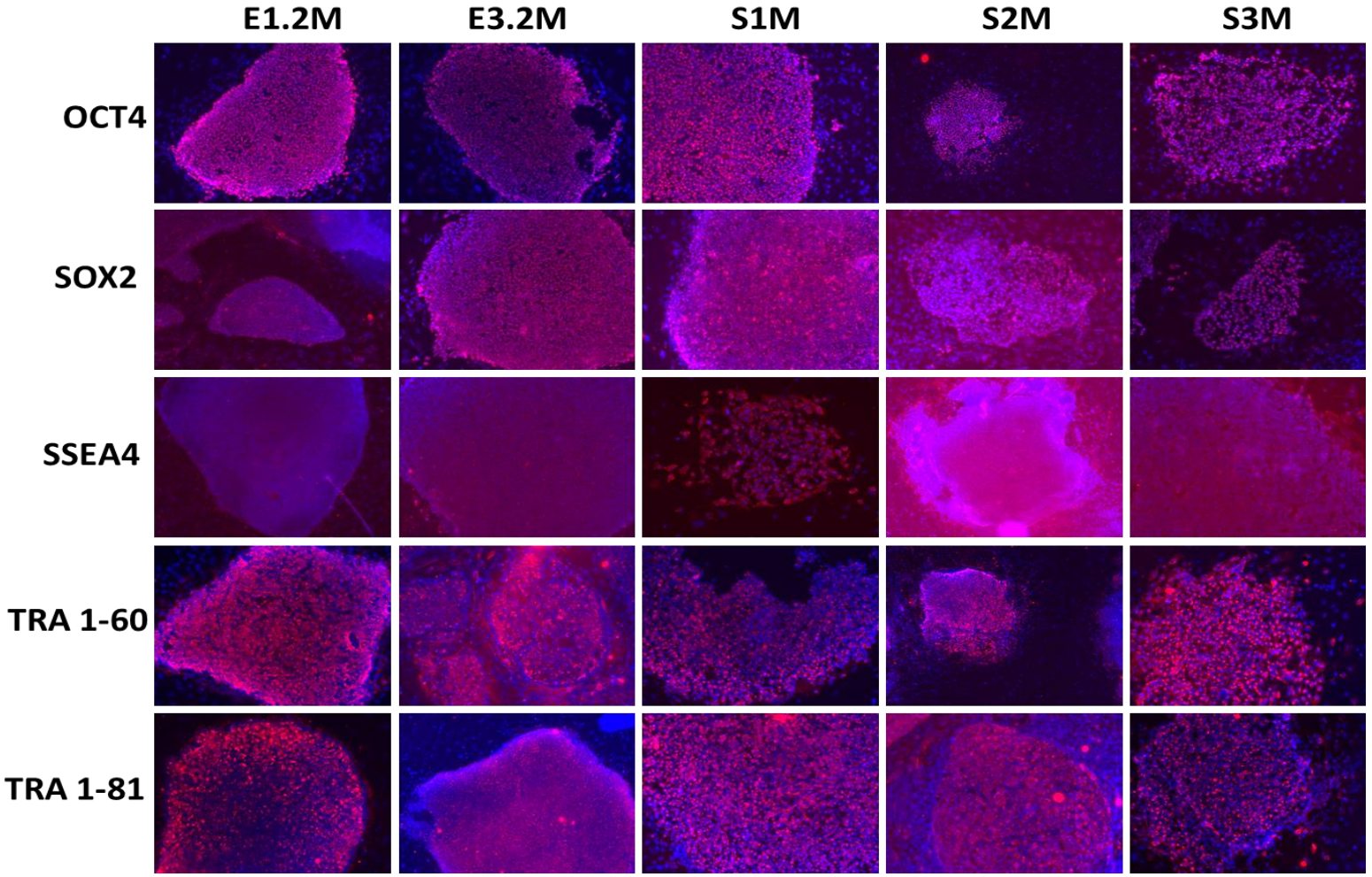
APPENDIX A



The episomal vectors used in this study. A) The episomal vectors used were the Y4 combination. B) the episomal plasmid charts. (Modified from Okita et al., 2011)

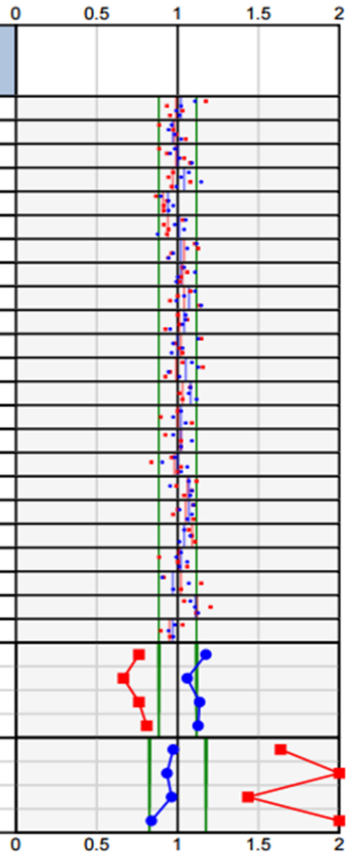
APPENDIX B



Immunocytochemistry for iPSC-lines E1.2, E2.3, S1M, S2M and S3M. Stained proteins included pluripotency markers Oct4, Sox2, SSEA4, TRA 1-60 and TRA 1-81. Only merge-images for each line are shown.

APPENDIX C

Probe	/F	/M	Chromosomal Location
01C	0.99	1.02	
02C	0.98	0.97	
03C	0.99	1.00	
04C	0.97	1.04	
05C	0.91	0.94	
06C	0.94	1.01	
07C	1.04	1.02	
08C	1.03	1.02	
09C	1.04	1.07	
10C	1.01	1.04	
11C	1.02	1.00	
12C	0.99	1.05	
13C	1.03	1.08	
14C	1.00	1.02	
15C	1.02	1.02	
16C	0.98	1.00	
17C	1.06	1.07	
18C	1.05	1.07	
19C	1.09	1.04	
20C	1.01	1.02	
21C	1.02	0.97	
22C	1.12	1.11	
Auto	0.95	0.97	
X1;ptel	0.76	1.17	-
X2;pcen	0.66	1.06	-
X3;qcen	0.76	1.14	-
X4;qtel	0.81	1.13	-
Y1;ptel	1.64	0.97	-
Y2;pcen	3.28	0.93	-
Y3;qcen	1.43	0.96	-
Y4;qtel	2.50	0.84	-



Karyotyping results for line E4G. The karyotype can be considered normal, if all probe values obtained for all chromosomes (except sex chromosomes) are 1 or close to one. The line E4G expressed a normal karyotype. (For more detailed description of the karyotyping analysis, see Lund et al. 2012)

APPENDIX D

	C-MYC	SOX2	KLF4	NANOG	GAPDH
E1.1M p. 3	22,3	19,3	23,8	21,5	16,7
E1.2M p. 3	22,1	20,1	24,2	20,8	16,9
E1.3M p. 3	22,8	20,1	24,9	21,4	17,5
E1.4M p. 3	21,7	20,0	23,9	21,2	17,1
E2.1M p. 3	21,9	19,6	24,3	21,1	17,3
E2.2M p.3	23,2	19,6	24,8	21,6	17,3
E2.3M p. 3	22,9	20,5	24,9	21,6	17,8
E2.4M p. 3	21,8	19,6	23,8	21,0	17,3
E3.1M p. 3	22,7	20,6	25,5	21,4	17,5
E3.2M p. 3	22,9	20,6	26,0	21,4	17,2
E1.1M p. 9	22,0	19,3	23,7	21,4	16,4
E1.2M p. 9	21,5	19,2	24,2	21,1	16,5
E1.3M p. 9	22,2	19,3	24,8	21,3	16,6
E1.4M p. 9	22,1	19,1	23,7	22,3	16,5
E2.1M p. 9	21,0	19,3	23,9	20,9	16,6
E2.2M p. 9	22,3	19,2	24,7	21,2	16,4
E2.3M p. 9	21,1	19,5	23,5	20,7	16,5
E3.1M p. 9	21,1	19,5	23,6	21,0	16,3
E3.2M p. 9	21,4	19,8	23,5	21,3	16,6

Ct-values determined using 7300 SDS Software (Applied Biosystems). Gene expression data for genes C-MYC, SOX2, KLF4 and GAPDH was obtained with a real-time-qPCR performed with the 7300 Real-time PCR system with Taqman chemistry using commercial probes. The values presented are means as each sample was prepared in triplicate.